AD NUMBER

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GRANT NUMBER DAMD17-94-J-4350

TITLE: Genes Involved in DNA Double-Strand Break Repair: Implications for Breast Cancer

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CONTRACTING ORGANIZATION: Stanford University
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REPORT DATE: October 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1996	Annual (1 Oct 95 - 30 Sep 96)

4. TITLE AND SUBTITLE
Genes Involved in DNA Double-Strand Break Repair: Implications for Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4350

6. AUTHOR(S)
Gilbert Chu, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Stanford University
Stanford, California 94305

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
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12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Ionizing radiation (IR) is both carcinogenic for breast tissue and used for treating breast cancer. In response to IR, cells undergo cell cycle arrest and repair the damaged DNA. To better combat breast cancer, the goal of this proposal has been to identify and characterize the cellular machinery required for this response.

We discovered that the Ku autoantigen is deficient in mutant cells from IR complementation group 5, which is defective in repair of the DNA double-strand breaks induced by IR or by V(D)J recombination. These mutant cells are rescued by an expression vector for the Ku86 subunit and contain mutations in the Ku86 gene. Thus, Ku is required for double-strand break repair.

In response to IR, cells transduce a signal that leads to accumulation of p53 and cell cycle arrest. One hypothesis is that the signal is mediated by DNA dependent protein kinase (DNA-PK), which consists of Ku as its regulatory subunit and a catalytic subunit (DNA-PKcs). The hypothesis was tested in primary fibroblasts from scid mice, which are deficient in DNA-PKcs. Scid cells showed normal p53 and cell cycle responses to IR. Thus, DNA-PK is not required for the induction of p53 or cell cycle arrest.

14. SUBJECT TERMS
Breast Cancer ionizing radiation, DNA repair, V(D)J recombination, cell cycle regulation, Ku, DNA-dependent protein kinase, p53

15. NUMBER OF PAGES
48

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

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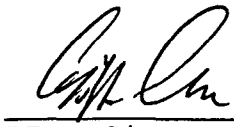

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Genes involved in DNA double-strand break repair: implications for breast cancer

I. Introduction

I.A. Nature of the problem

Ionizing radiation (IR) causes DNA damage by inducing a number of different lesions, including base damage and single-strand breaks. Double-strand breaks are especially significant, since they may lead to broken chromosomes and cell death if left unrepaired, or to chromosome aberrations and malignant transformation if repaired improperly.

The most important environmental source of exposure to IR is the decay of radium to form radon and then radon daughters. Radium is found in soil and rock and gaseous radon daughters will accumulate in homes, especially those built on land reclaimed from mining. Other sources of exposure include gamma rays from cosmic and terrestrial sources. Medical sources of exposure include diagnostic X-rays, nuclear medicine scans, and radiation therapy for cancer. Of note, IR is used in mammography screening for breast cancer, in radiation therapy for local treatment of newly diagnosed breast cancers, and in radiation therapy of metastatic breast cancer.

Ionizing radiation can cause cancers in many tissues, but breast tissue is particularly sensitive (Boice and Monson, 1976; Evans, et al., 1986). Mice exposed to IR show a dose-dependent increase in breast cancer (Ullrich, 1984). Humans exposed by the atomic bombs detonated over Hiroshima and Nagasaki had an increased incidence of breast cancer (Preston, et al., 1987). The risk was especially high if exposure occurred before age 10 and dropped significantly after age 40. Women who received radiation treatments for post partum mastitis also had an increased risk of breast cancer (Shore, et al., 1986).

Inherited diseases with marked predisposition to breast cancer include familial breast cancer, Li-Fraumeni syndrome, and ataxia telangiectasia (AT). AT is an autosomal recessive disease of particular interest here, because it links IR and breast cancer (Swift, et al., 1991). AT homozygotes are hypersensitive IR, and AT heterozygotes are reported to have a 5-fold risk for breast cancer. Since AT heterozygotes represent 1% of the general population, 5% of all breast cancers may be caused by inherited mutations in the AT gene.

Thus, IR is implicated in the development of breast cancer, employed in breast cancer screening, and used for breast cancer treatment. A better understanding of the causes and treatment of breast cancer would be gained by studying how cells repair damage from IR. However, until recently, the genes involved in repairing the DNA double-strand breaks caused by IR had not been identified. The overall goal of this proposal was to identify such genes.

I.B. Background of previous work

We previously identified a DNA end-binding (DEB) factor that bound specifically to double-stranded DNA ends and was deficient in three mutant cell lines that were hypersensitive to IR (Rathmell and Chu, 1994a). All three cell lines belonged to the same genetic complementation group (X-ray group 5), suggesting that they were all defective in the same gene product.

Cells from group 5 as well as those from groups 4 and 7 are sensitive to IR because of a basic defect in repairing DNA double-strand breaks (Pergola, et al., 1993; Taccioli, et al., 1993). Interestingly, these cells are also defective in their capacity to activate a process known as V(D)J recombination, which is the mechanism used by the immune system to generate a diverse array of recognition molecules consisting of antibodies produced by B cells and receptors on the surface of T cells.

Because cells from groups 4, 5, and 7 are defective for both repair of IR damage and V(D)J recombination, the same DNA rejoining machinery appears to be used for both processes. The importance of this machinery is exemplified in the scid mouse, an animal characterized by severe combined immunodeficiency, sensitivity to IR, and extreme susceptibility to lymphomas (Bosma and Carroll, 1991).

By characterizing DEB activity, we noted a number of strong similarities to a well studied protein, the Ku autoantigen. Ku was originally identified as a major antibody target in patients with several autoimmune diseases, including scleroderma, polymyositis, systemic lupus, and Grave's disease (Mimori, et al., 1981). Ku is a protein with two subunits of 70 kDa and 86 kDa

(Ku 70 and Ku86). The cDNAs for both subunits had been cloned previously. Both DEB activity and Ku bound to double-stranded but not single-stranded DNA ends. Both were localized to the nucleus of cells and moderately abundant, with 200,000 to 400,000 molecules per cell. Despite 15 years of investigation, the normal function of Ku in the cell remained obscure (Reeves, 1992).

To investigate the possible identity of DEB factor and Ku, two different human Ku antisera were added to the binding reaction containing cell extract and probe DNA. Both antisera cross-reacted with DEB factor in an electrophoretic mobility shift assay (Rathmell and Chu, 1994b). The antisera also showed that a polypeptide with the antigenic determinants of Ku70 co-fractionated with DEB activity on a heparin agarose column. Furthermore, an expression vector for hamster Ku86 cDNA fully restored both IR resistance and V(D)J recombination to the group 5 mutant hamster cells (Smider, et al., 1994; Taccioli, et al., 1994). Finally, in collaboration with Margaret Zdzienicka's lab, we found that two different mutant cell lines from group 5 contained two different internal in-frame deletions in the open reading frame of Ku86 (Errami, et al., 1996). (These results were presented in our annual report for 1995.)

I.C. Purpose of the present work

The purpose of the work described in the report was to answer the following questions.

1. What is the role of Ku in yeast?
2. Is there a cell line mutant for Ku70?
3. How is DNA-PK assembled at DNA ends to facilitate double-strand break repair?
4. Does DNA-PK play a role in the p53-dependent signaling pathway for cell cycle arrest?

Our progress in addressing these questions is described below.

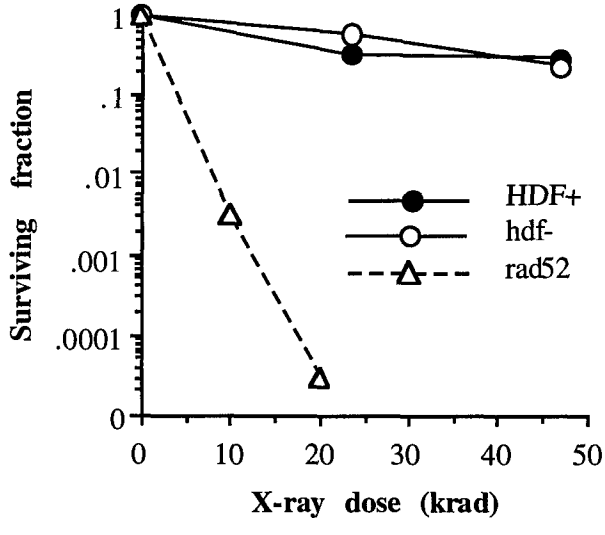
II. Experimental methods and results

II.A. What is the role of Ku in yeast?

Feldmann and Winnacker identified and purified a DNA end-binding factor in the yeast Saccharomyces cerevisiae (Feldmann and Winnacker, 1993). The purified protein consisted of two polypeptides of about 70 and 80 kDa, and amino acid sequences from the 70 kDa subunit permitted the isolation of a gene that proved to be homologous to mammalian Ku70. We obtained both the yeast gene (HDF) and a yeast strain deleted for the homolog of Ku70 (hdf-) as a kind gift from Dr. Winnacker. To see if Ku in yeast might have the same role in double-strand break repair as it has in mammalian cells, we tested the yeast hdf- strain for its sensitivity to IR (Figure 1).

Fig. 1. Yeast hdf- mutants have wild type survival after IR.

Yeast mutant in a homolog of mammalian Ku70 (hdf-) lacked DNA end-binding activity. They were tested for survival after exposure to different doses of IR. For comparison, the mutant yeast strain rescued with the HDF gene (HDF+) and a yeast strain mutant for rad52 were also tested.



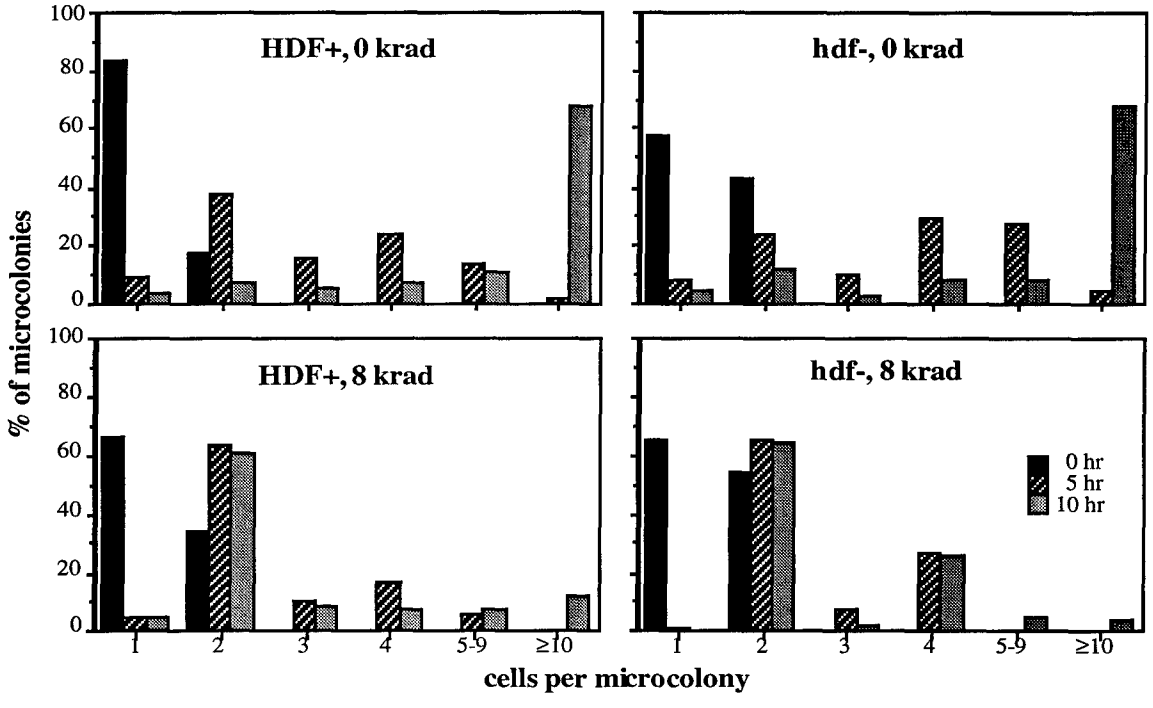
Unlike mammalian Ku mutants, the yeast Ku mutant had wild type survival after exposure to IR. The reason for this difference between yeast and mammals is that the dominant pathway for

double-strand break repair in yeast involves homologous recombination, while the dominant pathway in mammals involves non-homologous end-joining. Thus, in a control experiment, a rad52 mutant, which is defective in homologous recombination, was hypersensitive to IR, as previously described (Fig.1). Recent results by another laboratory suggest that yeast Ku nevertheless plays a role in joining free DNA ends (Milne, et al., 1996). We are currently collaborating with Michael Fasullo's lab to study this Ku-dependent end-joining reaction further by engineering an HO endonuclease recognition site into a chromosomal locus. In the planned experiment, the induction of HO endonuclease would lead to cleavage of the chromosome carrying the recognition site. We will then be able to study the end-joining reaction in wild type and Ku mutant yeast strains.

The cellular response to DNA double-strand breaks includes arrest of the cell cycle as well as repair of the damaged DNA. To determine whether yeast Ku is involved in cell cycle arrest, hdf- mutants were compared to wild type yeast after exposure to IR (Fig. 2). No difference was seen, demonstrating that Ku in yeast is not required for cell cycle arrest after IR.

Fig. 2. Yeast hdf- mutants have wild type cell cycle arrest after IR.

Wild type (HDF+) and mutant (hdf-) yeast were spread on solid media and either left unexposed or exposed to 8 krad of IR. Microcolonies were then enumerated at 0, 5, and 10 hr. Yeast not exposed to IR continued to pass through the cell cycle, leading to a change in the distribution towards microcolonies with a greater number of cells. By contrast, yeast exposed to IR underwent cell cycle arrest, so that the microcolonies accumulated at 2 cell stage, which corresponded to arrest of the cells in the form of a single-budded cell. The same degree of cell cycle arrest was seen for both wild type and mutant strains.



II.B. Is there a mammalian cell line mutant for Ku70?

We recently became aware of a mutant Chinese hamster ovary cell line, SRD-1, characterized by Brown and Goldstein for the study of cholesterol biosynthesis (Yang, et al., 1994). These cell lines fail to repress sterol synthesis when incubated with 25-hydroxycholesterol due to a rearrangement in the gene encoding SREBP-2, a transcription factor that regulates cholesterol homeostasis. The rearrangement proved to be a chromosomal translocation that truncates SREBP-2 by juxtaposition to and disruption of the Ku70 locus. The disruption of one of

the two Ku alleles might lead to a phenotype, since Chinese hamster ovary cells are functionally hemizygous in large parts of their genome due to methylation silencing. Therefore, to determine whether this translocation led to the loss of Ku binding activity, we obtained the SRD-1 cell line as a kind gift from Dr. Goldstein.

Our preliminary results indicate that SRD-1 lacks Ku DNA-binding activity. In contrast, binding activity was present in the cell line SRD-2, which involves the translocation of the SREBP-2 gene to another, previously uncharacterized gene. Current experiments aim to determine if the SRD-1 cell line is X-ray sensitive and defective in V(D)J recombination and if the phenotype can be rescued by transfection of an expression vector for Ku70. This cell line would then be the first known Ku70 mutant, providing a valuable reagent for further study of Ku70. The availability of mutant cell lines for both Ku70 and Ku86 will permit mutagenesis studies to determine the regions in the Ku genes that encode its cellular and biochemical properties.

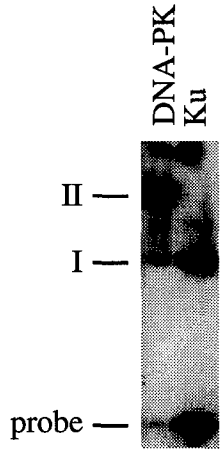
II.C. How is DNA-PK assembled at DNA ends to facilitate double-strand break repair?

The work described above identified four genes involved in a common biochemical pathway for rejoining free DNA ends shared by DSB repair and V(D)J recombination. To study this pathway biochemically, we have developed several in vitro assays that can be performed on crude extracts of cells.

We previously developed an electrophoretic mobility shift assay (EMSA) for measuring Ku binding to DNA ends. This assay was extremely sensitive for Ku but failed to reveal the assembly of the full DNA-PK enzymatic complex on DNA ends. We therefore developed a new EMSA that would circumvent this problem. In the new EMSA, extracts were incubated with a short DNA probe of only 32 bp. We discovered that the short DNA probe was not affected by nonspecific DNA binding proteins, and so supercoiled competitor DNA could be omitted from the binding reaction. Under these conditions, the EMSA detected a new complex of very low mobility corresponding to the assembly of a complex of DNA, Ku, and DNA-PKcs.

Figure 3 shows data demonstrating successful detection of the DNA-PK complex. Incubation of the short DNA probe with purified Ku produced only a high mobility protein-DNA complex, while incubation with purified DNA-PK produced both high and low mobility complexes. In addition, incubation of the probe with HeLa extracts produced both high and low mobility complexes, and the addition of three different monoclonal antibodies against different epitopes of DNA-PKcs either supershifted or disrupted the low mobility complex. Furthermore, antibodies against Ku supershifted both high and low mobility complexes (V. Smider and G. Chu, unpublished data). Finally, incubation of the probe with extracts from scid cells, which are known to be normal for Ku binding activity but defective in DNA-PK enzymatic activity, produced a normal high mobility complex and a grossly abnormal spectrum of low mobility complexes that appeared as a broad smear on the gel (V. Smider and G. Chu, unpublished data).

Fig. 3. EMSA for assembly of DNA-PK.
The 32P-labeled 32 bp DNA probe was incubated with purified DNA-PK (containing DNA-PKcs and both subunits of Ku) or with purified Ku alone (consisting of both subunits of Ku). After 10 min. at room temperature, the products were resolved by electrophoresis in nondenaturing polyacrylamide gel and then visualized by autoradiography. The position of the free DNA probe is shown at the bottom of the gel. Band I shows the shifted mobility of the DNA probe due to the formation of a Ku/DNA complex. Band II shows the shifted mobility of the DNA probe due to the formation of the higher order Ku/DNA-PKcs/DNA complex.



The kinase activity of DNA-PK was measured by incubating cell extracts with 32P-γ-labeled ATP and a peptide containing a sequence recognized specifically by DNA-PK but not by other cellular kinases, EPPLSQEAFADLWKK. This peptide contains an SQ motif that is the preferred site for serine phosphorylation by DNA-PK. After incubation, the mixture was passed over a nitrocellulose filter, which was then assayed for 32P-phosphorylated peptide in a scintillation counter. The signature of DNA-PK enzymatic activity was phosphorylation of the peptide that depended on the addition of DNA ends. In adapting this assay for our laboratory, we discovered that precipitation of very high molecular weight complexes with polyethylene glycol improved the sensitivity of the DNA-PK enzymatic assay.

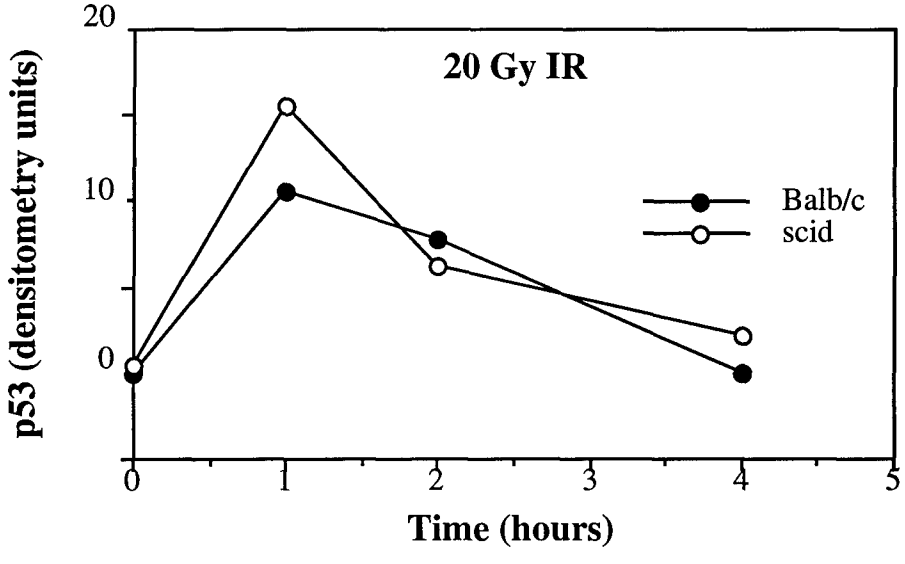
These assays enable us to measure both the assembly and enzymatic activity of DNA-PK due to its interaction with DNA ends. We are now using these assays to better understand how Ku and DNA-PK interact with DNA to facilitate the DNA end-joining reaction. We are also using these assays to determine whether DNA-PK is defective in breast cancer patients who have suffered adverse reactions to radiation therapy.

II.D. Does DNA-PK play a role in the p53-dependent signaling pathway for cell cycle arrest?

In response to DNA damage, cells transduce a signal that leads to accumulation of p53, induction of several genes, including p21, gadd45, and gadd153, and cell cycle arrest. One hypothesis is that the signal is mediated by DNA dependent protein kinase (DNA-PK), which consists of a catalytic subunit (DNA-PKcs) and a regulatory subunit (Ku). DNA-PK has several characteristics that support this hypothesis: Ku binds to DNA damaged by nicks or double-strand breaks, DNA-PKcs is activated when Ku binds to DNA, DNA-PK will phosphorylate p53 and other cell cycle regulatory proteins in vitro, and DNA-PKcs shares homology with ATM, which is mutated in ataxia telangiectasia and involved in signaling the p53 response to IR.

The hypothesis was tested by analyzing early passage fibroblasts from scid mice, which are deficient in DNA-PK (Rathmell, et al., 1996). Scid and wild type cells exposed to IR and ultraviolet radiation showed the same accumulation of p53. To fully explore any possible abnormality in scid cells, the p53 response was measured at different times and for different doses of radiation. Representative data for IR are shown in Figure 4.

Fig. 4. p53 induction after IR in scid and wild type cells



The induction of *p21, gadd45,* and *gadd153* also occurred normally in scid cells after exposure to ultraviolet radiation, IR, or methyl methanesulfonate. Finally, in collaboration with the lab of Dr. William Kaufmann, we found that both G1 and G2 cell cycle arrest occurred normally after scid cells were exposed to IR (Tables 1 and 2).

Table 1. G_1 checkpoint after ionizing radiation in scid and wild type cells

The cells were analyzed for G_1 arrest 6-8 h after exposure to 8 Gy of IR. The table shows the relative numbers of diploid cells in the first (2-3N) and second (3-4N) halves of S phase and depletion of the cells in the first half of S phase (G_1 arrest). The data for 2-3N and 3-4N are given as a percent of mock-treated control cells. Cells analyzed were wild type Balb/c and agouti mouse cells, scid mouse cells, and normal human fibroblasts. The numbers of independent experiments are shown in parentheses.

Cells	2-3N	3-4N	G_1 arrest
Balb/c (n=2)	33%	112%	67%
agouti (n=2)	43%	106%	57%
scid (n=3)	31%	98%	69%
human (n=1)	4%	152%	96%

Table 2. G_2 checkpoint after ionizing radiation in scid and wild type cells

The cells were analyzed for G_2 arrest after exposure to 2 and 4 Gy of IR. The table shows the fraction of cells in mitosis as a percentage of mock-treated control cells 2 h after exposure. The numbers of independent experiments are shown in parentheses.

Cells	2 Gy	4 Gy
Balb/c	22% (n=2)	0% (n=1)
agouti	14% (n=1)	0% (n=1)
scid	14% (n=5)	1% (n=4)

In summary, after exposure to ionizing radiation, ultraviolet radiation, and methyl methanesulfonate, scid and wild type cells were indistinguishable in their response. The accumulation of p53, induction of p21, gadd45, and gadd153, and arrest of the cell cycle in G_1 and G_2 occurred normally. Therefore, DNA-PK is not required for accumulation of p53 or cell cycle arrest following DNA damage.

III. Conclusions

Several major conclusions arise from our studies.

1. Ku is essential for both DNA double-strand break repair and V(D)J recombination.
   The identification of Ku as a component in these pathways will stimulate progress in understanding the biochemistry of these important processes, since a great deal was already known about the biochemistry of Ku and its associated DNA-dependent protein kinase. As described in the introduction, better understanding of the causes and treatment of breast cancer will be gained by elucidation of how cells repair damage from IR.
2. DNA double-strand break repair and V(D)J recombination share a common biochemical pathway for joining free DNA ends.
   Because of the shared biochemical pathway, insights gained for one process will be relevant for the other. Furthermore, this discovery suggests that evolution of the V(D)J recombination pathway involved the hijacking of a pre-existing DNA repair pathway for a new role in the immune system.
3. The Ku86 gene is mutated in cells from X-ray complementation group 5.
   The discovery that the primary genetic defect in these cells is in the Ku86 gene means that these cells will be an invaluable reagent for further studies on the cellular roles of Ku. Hypotheses for additional roles of Ku can and have been tested in these cells. Furthermore, transfection of the cells with mutated versions of the Ku genes will allow us to dissect the regions of Ku required for its different roles in repair and recombination.
4. DNA-PK is not required for p53 induction or cell cycle arrest after DNA damage.
   These findings restrict the possible role of DNA-PK as the long sought signaling molecule for transducing a signal from damaged DNA to p53. Therefore the search for this signaling molecule must be redirected towards other candidates.

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VI. Personnel receiving pay from this effort

Gilbert Chu, principal investigator
W. Kimryn Rathmell, predoctoral student
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VII. Appendices

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MOLECULAR AND CELLULAR BIOLOGY, Apr. 1996, p. 1519–1526
0270-7306/96/$04.00+0

Vol. 16, No. 4

Ku86 Defines the Genetic Defect and Restores X-Ray Resistance and V(D)J Recombination to Complementation Group 5 Hamster Cell Mutants

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Received 1 November 1995/Returned for modification 28 December 1995/Accepted 19 January 1996

X-ray-sensitive hamster cells in complementation groups 4, 5, 6, and 7 are impaired for both double-strand break repair and V(D)J recombination. Here we show that in two mutant cell lines (XR-V15B and XR-V9B) from group 5, the genetic defects are in the gene encoding the 86-kDa subunit of the Ku autoantigen, a nuclear protein that binds to double-stranded DNA ends. These mutants express Ku86 mRNA containing deletions of 138 and 252 bp, respectively, and the encoded proteins contain internal, in-frame deletions of 46 and 84 amino acids. Two X-ray-resistant revertants of XR-V15B expressed two Ku86 transcripts, one with and one without the deletion, suggesting that reversion occurred by activation of a silent wild-type allele. Transfection of full-length cDNA encoding hamster Ku86 into XR-V15B cells resulted in a complete rescue of DNA-end-binding (DEB) activity and Ku70 levels, suggesting that Ku86 stabilizes the Ku70 polypeptide. In addition, cells expressing wild-type levels of DEB activity were fully rescued for X-ray resistance and V(D)J recombination, whereas cells expressing lower levels of DEB activity were only partially rescued. Thus, Ku is an essential component of the pathway(s) utilized for the resolution of DNA double-strand breaks induced by either X rays or V(D)J recombination, and mutations in the *Ku86* gene are responsible for the phenotype of group 5 cells.

All cells possess a mechanism for repairing DNA double-strand breaks (DSBs) produced by ionizing radiation. Cells of the immune system must also resolve DNA DSBs produced by V(D)J recombination of the immunoglobulin and T-cell receptor genes during development of B and T cells (reviewed in reference 28). In fact, the biochemical pathways for DSB repair and V(D)J recombination have a number of common factors. Evidence of this was first described for the severe combined immune deficient (*scid*) mouse, which is hypersensitive to ionizing radiation because of defective DSB repair (1, 12, 18) and immune deficient because of defective V(D)J recombination (29). Subsequently, other X-ray-sensitive rodent cell lines defective in DSB repair were also found to have impaired V(D)J recombination (17, 27, 34, 45, 47). These cell lines fall into four complementation groups, 4, 5, 6, and 7, with group 7 corresponding to the *scid* defect (43, 52). The human genes capable of rescuing these mutants are designated *XRCC4*, *XRCC5*, *XRCC6*, and *XRCC7*, respectively (*XRCC* denotes X ray cross-complementing) (for a review, see reference 51).

Recently, it has been shown that the Ku protein is involved in both DSB repair and V(D)J recombination. Ku is an abundant nuclear protein identified originally as an autoantigen recognized by sera from patients with autoimmune diseases, including scleroderma-polymyositis overlap syndrome and systemic lupus erythematosus (31). Ku is a heterodimer of two tightly associated polypeptides of 70 and 86 kDa (Ku70 and Ku86, respectively) encoded by genes which have been mapped in humans to chromosomes 22q13 and 2q33-35, respectively (4, 32, 38). Biochemically, it has been shown that Ku binds to double-stranded DNA ends, nicks, gaps, and DNA hairpins (30). It also forms a complex with a catalytic subunit of 450 kDa to form the DNA-dependent protein kinase. In vitro, the DNA-dependent protein kinase phosphorylates several proteins, such as RNA polymerase II, Sp1, p53, c-Fos, c-Jun, c-Myc, and Ku itself (20). The gene encoding the catalytic subunit (14) maps to human chromosome 8q11 (19, 24–26) and has recently been shown to rescue the *scid* defect (2, 23, 35).

There is indirect evidence that mutation of the *Ku86* gene might be the primary defect in complementation group 5 mutants. The *XRCC5* gene has been mapped to 2q35, the same region as that of the *Ku86* gene (6, 16, 21). In addition, group 5 cells lack a DNA-end-binding (DEB) activity with an abundance, nuclear localization, DNA substrate specificities, and antigenic determinants similar to those of Ku (13, 36, 37). Transfection of the human Ku86 cDNA into group 5 cells partially restores DEB activity, X-ray resistance, and V(D)J recombination activity in different mutants belonging to this group (3, 42, 44).

Nevertheless, direct evidence defining the primary defect in mutants of group 5 was still lacking. First, human Ku86 cDNA did not completely rescue group 5 mutants. Second, chromosome mapping experiments sampled only a fraction of the human genome (6, 21) and therefore failed to rule out the possibility that complementing activity was present on another chromosome in addition to chromosome 2. Therefore, we in-

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troduced a wild-type hamster cDNA into group 5 mutant cells and demonstrated that this could completely restore DEB activity, Ku70 protein levels, X-ray resistance, and V(D)J recombination. Furthermore, we isolated cDNAs encoding Ku86 from two independently derived group 5 mutants and demonstrated that these cDNAs are mutated. These results unequivocally demonstrate that mutation of Ku86 is directly responsible for the defects exhibited by XRCC5 cells and confirm the idea that Ku86 is a critical component of the mammalian DNA DSB repair pathway.

MATERIALS AND METHODS

Cell culture. XR-V15B and XR-V9B mutant cell lines derived from Chinese hamster V79 and V79B cells, respectively, have been described previously (53, 54). All cell lines were cultured in Ham's F-10 medium (without hypoxanthine and thymidine) supplemented with 15% newborn calf serum or in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% L-glutamine. Culture media also included penicillin (100 U/ml) and streptomycin (0.1 mg/ml). Cells were maintained at 37°C in a 5% CO_2 atmosphere, humidified to 95 to 100%.

To isolate X-ray-resistant revertants, XR-V15B and a thioguanine- and ouabain-resistant derivative (XR-V15BTOR) cell line were seeded (at 10^6 cells per 10-cm-diameter plate) and exposed to an X-ray dose of 2 Gy for three consecutive days. Clones of cells surviving this selection were isolated and examined for X-ray survival. Two revertants (XR-V15B-Rev1 and XR-V15B-Rev2) from XR-V15B and one revertant (XR-V15B-Rev3) from XR-V15BTOR showed wild-type X-ray survival.

DNA transfection. Syrian hamster Ku86 cDNA inserted into the pSP65-SRα vector under the control of the SRα promoter (46) or the same vector lacking this insert (10 μg) was cotransfected with pRSVneo (2 μg) and carrier DNA consisting of pUC19 (8 μg) by coprecipitation with calcium phosphate in 0.8 ml onto a 10-cm-diameter plate containing 10^6 cells (15). After 48 h, cells were passaged and then selected for 14 days in medium containing G418 (Gibco, Gaithersburg, Md.) at 400 μg/ml. Colonies stably resistant to G418 were isolated and characterized as described below.

Electrophoretic mobility shift assay. Nuclear extracts were prepared by the Nonidet P-40 lysis method previously described (36). Extract (0.6 μg) was incubated with radiolabeled f148 probe in the presence of closed circular plasmid DNA to mask the effect of nonspecific DNA-binding proteins. Protein-DNA complexes were resolved on a nondenaturing 5% polyacrylamide gel.

Immunoblotting. Nuclear extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane (GSWP; Millipore, Bedford, Mass.), and probed with human OM antiserum (a kind gift from John Hardin, 1:2,000 dilution) followed by horseradish peroxidase-conjugated goat antibody to human immunoglobulin G (TAGO, Burlingame, Calif.). Antibody binding was detected by enhanced chemiluminescence (Amersham, Arlington Heights, Ill.).

X-ray survival. G418-resistant clones were plated in triplicate and X-irradiated at doses ranging from 0 to 6 Gy. After 14 days, surviving colonies were stained with 10% Giemsa stain and counted. Each survival curve represents the average of two independent experiments.

V(D)J recombination. G418-resistant clones were removed from G418 on the day prior to beginning the assay. Expression plasmids for RAG-1 and RAG-2 (8 μg each) were transiently transfected with either pJH290 (4 μg) to test for coding joint formation or pJH200 (4 μg) to test for signal joint formation. DNA was transfected by coprecipitation with calcium phosphate. After 48 h, DNA was harvested, digested with *Dpn*I to eliminate unreplicated plasmids, and electroporated into *Escherichia coli*. Unrearranged plasmids conferred resistance to ampicillin (Amp^r), whereas rearranged plasmids conferred resistance to both ampicillin and chloramphenicol (Amp^r Cam^r). Recombination frequency is expressed as the ratio of doubly drug-resistant colonies to the number of ampicillin-resistant colonies. Valid V(D)J recombination events were confirmed by digesting plasmids recovered from *E. coli* with *Apa*LI (which cleaves at the signal joint) or *Pvu*II (which releases a fragment spanning a correctly formed coding joint) (34).

RT-PCR analysis of hamster Ku86 mRNAs from wild-type and mutant cells. Total RNA was isolated by the guanidium isothiocyanate method previously described (7). First-strand Ku86 cDNA synthesis was performed as follows: total RNA (1 μg) was added to a reverse transcription (RT) solution consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 mM (each) deoxynucleoside triphosphates, 2.5 μM oligo$(dT)_{15}$ primer, 20 U of RNasin, and 50 U of murine leukemia virus reverse transcriptase (Geneamp RNA PCR kit). The reaction mixture (final volume, 20 μl) was incubated for 30 min at 42°C, heated to 95°C for 5 min, and then chilled on ice. The newly generated RNA-cDNA hybrids were amplified by PCR with Ku86-specific primers Ku-5 and Ku-3b, resulting in a 2.3-kb product. PCR was performed with cycles consisting of denaturation for 30 s at 94°C, primer annealing at 55°C for 30 s, and extension at 72°C for 2 min (DNA thermal cycler; Perkin-Elmer Cetus, Norwalk, Conn.).

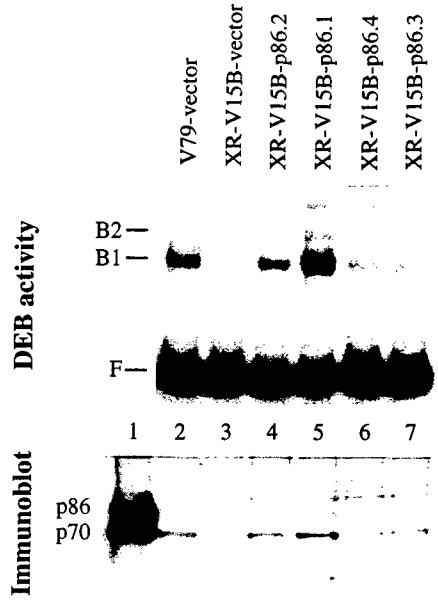

FIG. 1. Expression of Ku in wild-type V79 and mutant XR-V15B hampster cells transfected with Ku86. (Top) DEB activity. The levels of DEB activity in one clone isolated from the wild-type hamster cell line V79 transformed with vector alone (V79-vector; lane 2), one clone isolated from the transfection of XR-V15B with vector alone (XR-V15B-vector; lane 3), and four clones of mutant XR-V15B cells transfected with hamster Ku86 (XR-V15B-p86.1, XR-V15B-p86.2, XR-V15B-p86.3, and XR-V15B-p86.4; lanes 4 to 7) are shown. Clones are numbered in order of decreasing DEB activity. F, positions of free DNA probe; B1 and B2, positions of protein-DNA complexes consisting of DEB activity and the DNA probe. (Bottom) Immunoblot for expression of Ku70. The immunoblot was probed with OM antiserum, which reacts strongly with both Ku86 and Ku70 in human cells and less strongly with Ku70 in hamster cells. The expression of Ku70 in the hamster cell lines used for the upper panel is shown. Lane 1 is a control with HeLa extract to show the mobilities of human Ku70 and Ku86 (p70 and p86, respectively).

DNA sequence analysis. Primer Ku-3b was biotinylated, and after PCR, the amplified 2.3-kb cDNA was gel purified (Qiaex; Qiagen, Chatsworth, Calif.). Magnetic M-280 Dynabeads (Dynal AS, Oslo, Norway) were used to prepare single-stranded, immobilized templates. The DNA sequence was obtained by using a T7 sequence kit (Pharmacia Biotech, Uppsala, Sweden) with [α-^{32}P] dATP. Samples were resolved on a 6% polyacrylamide gel at 40 W. Gels were exposed to X-ray film. Sequence analysis was also performed after cloning the PCR products in a TA cloning vector (Promega, Madison, Wis.). The primer sequences were as follows: Ku-2, 5′-GGAGGAGGCCATCCAGTT-3′; Ku-3b, 5′-ATGGCTTCCAGGATGCTCTTTC-3′; Ku-4, 5′-TGATGCTACCAGATTTTG-3′; Ku-5, 5′-AAGTAACCAAACCGCCCGTG-3′; Ku-6, 5′-ATTGTAGCCTATAAATCG-3′; Ku-7, 5′-CCGTAGCGGAACCCTTGAAT-3′; Ku-8, 5′-AGAGCTAATCCTCAAGTTGG-3′; Ku-8b, 5′-GGTTGAACTCCTAATCGAGA-3′; Ku-9, 5′-ACTCCATCCTGAACAACAAT-3′; Ku-10, 5′-ATTCAGCAGCACATTTTG-3′; Ku-12, 5′-CAAAATGTGCTGCTGAAT-3′; and Ku-13, 5′-CTTTGCAGCAAAAGATGAT-3′.

Nucleotide sequence accession numbers. The Chinese hamster Ku86 sequence has been deposited in the GenBank database under accession number L48606. The GenBank and EMBL accession numbers for the Syrian hamster, mouse, and human Ku86 nucleotide sequences are U40570, X66323, and M30938, respectively.

RESULTS

Restoration of DEB activity. A hamster cDNA encoding Ku86 had been cloned previously from a Syrian hamster liver library (17). Therefore, XR-V15B mutant cells were transfected either with expression vectors containing the Syrian hamster Ku86 or with a vector lacking this insert. As a control, the parental, wild-type V79 cell line was also transfected with a vector lacking this insert. Stable G418-resistant clones were isolated and analyzed for DEB activity. Of 12 clones selected in G418 from XR-V15B cells transfected with Ku86, 5 expressed

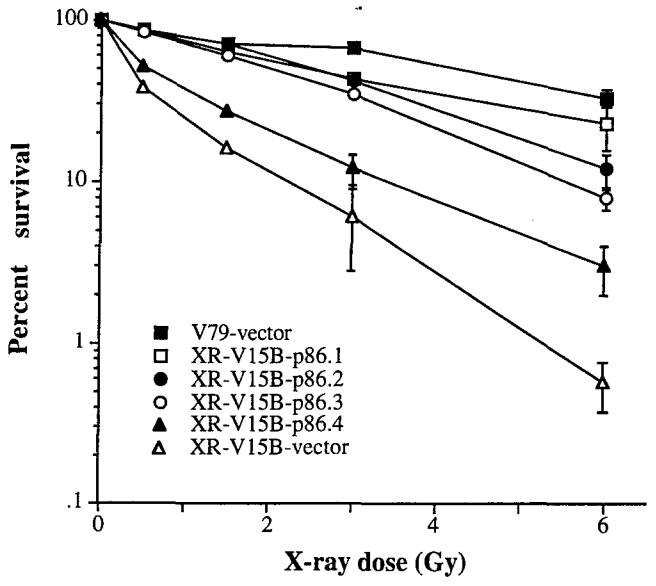

FIG. 2. X-ray survival of XR-V15B cells transfected with hamster Ku86 cDNA. Clones are labeled as in Fig. 1. Single cells were plated at low density and X-irradiated at various doses, and then the percentages of cells surviving to form colonies at 14 days postirradiation were determined. Data are the averages of two experiments, each performed in triplicate. Vertical bars show standard deviations.

detectable DEB activities. Four of these clones were chosen for further analysis because they showed a range of DEB activities, from below to above wild-type levels (Fig. 1). The clones were designated according to their levels of DEB activity from highest to lowest, XR-V15B-p86.1, XR-V15B-p86.2, XR-V15B-p86.3, and XR-V15B-p86.4. Of the nine clones isolated from XR-V15B cells transfected with vector alone, none showed DEB activity. Thus, the probability of finding spontaneous revertants among transfected XR-V15B cells was too low to account for the five clones with detectable DEB activities.

Restoration of Ku70 protein level. It was previously shown that group 5 cells lack Ku70 protein, even though the group 5 defect did not map to the same chromosome as that of the *Ku70* gene (37, 42). Therefore, we investigated the status of Ku70 expression in stably transfected clones. Complete restoration of Ku70 protein to wild-type levels was observed by immunoblot with Ku antiserum in the clone expressing the highest level of DEB activity (XR-V15B-p86.1) (Fig. 1). Smaller increases in Ku70 protein levels were seen for clones which expressed lower levels of DEB activity (XR-V15B-p86.2, XR-V15B-p86.3, and XR-V15B-p86.4). Very little, if any, detectable Ku70 protein was detected in a cloned line transfected with vector alone (XR-V15B-vector). Thus, the introduction of Ku86 cDNA to XR-V15B cells restored Ku70 accumulation.

Complementation of X-ray sensitivity. Previous studies demonstrated that the introduction of human Ku86 cDNA into the hamster XR-V15B cell line partially complemented its X-ray sensitivity (42). To test if complete complementation could be obtained with hamster cDNA, we tested our transfected clones for X-ray resistance. Complete restoration of X-ray resistance was attained in clone XR-V15B-p86.1, which expressed the highest level of DEB activity (Fig. 2). Only partial, albeit significant, restoration of X-ray resistance was seen in clones expressing lower levels of DEB activity (Fig. 2). Thus, hamster Ku86 cDNA can fully complement the X-ray sensitivity of group 5 cells; this is dependent on the level of DEB activity.

Complementation of V(D)J recombination. Group 5 cells are defective in forming coding and signal joints during V(D)J recombination (34, 45). Therefore, we tested our transfected clones for proficiency in V(D)J recombination by measuring both signal and coding joint formation in an extrachromosomal assay (42). V(D)J recombination was measured for both signal and coding joint formation, and valid recombination events were confirmed by restriction analysis. Clones expressing the highest levels of DEB activity also had the highest levels of V(D)J recombination activity (Fig. 3). Within the expected uncertainty of this assay, clone XR-V15B-p86.1 showed wild-type or nearly wild-type levels of V(D)J recombination. Interestingly, in each of the partially rescued clones, the relative restoration of coding joint formation was less efficient than that of signal joint formation. This suggests that full Ku expression is more important for coding joint formation. In summary, the introduction of hamster Ku86 cDNA into XR-V15B cells restored DEB activity, Ku70 expression, X-ray sensitivity, and V(D)J recombination. The level of restoration in each case

Cell Line	Amp^R	Amp^RCam^R	RF (10^{-3})
	Signal joints		
V79-vector	31800	32	1.01
V15B-p86.1	55000	44	.80
V15B-p86.2	79900	42	.53
V15B-p86.3	77500	34	.44
V15B-p86.4	80400	34	.43
V15B-vector	86600	0	<.01
	Coding joints		
V79-vector	9335	60	6.43
V15B-p86.1	13480	60	4.45
V15B-p86.2	36800	48	1.30
V15B-p86.3	14000	24	1.71
V15B-p86.4	18500	8	.43
V15B-vector	46600	0	<.02

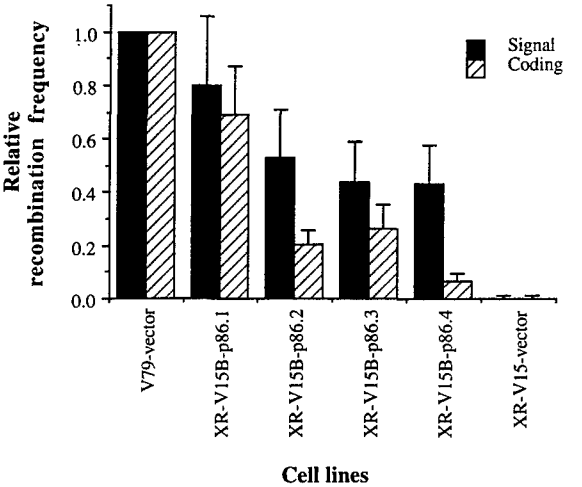

FIG. 3. V(D)J recombination activity of XR-V15B cells transfected with hamster Ku86 cDNA. Clones are labeled as in Fig. 1. Cell lines were transfected with the *RAG-1* and *RAG-2* genes along with a substrate plasmid (see Materials and Methods). The V(D)J recombination frequency (RF) was measured for both signal and coding joints as the number of doubly resistant (Amp^r Cam^r) colonies divided by the number of Amp^r colonies. Vertical bars show standard deviations.

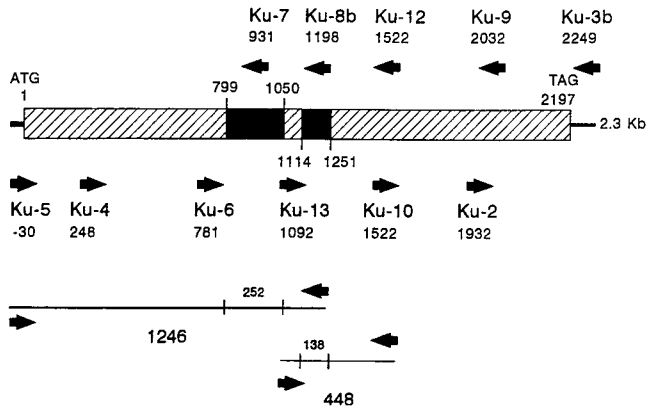

FIG. 4. Hamster Ku86 cDNA and PCR primers. The positions of the primers used for sequencing and RT-PCR are indicated by arrows. Black boxes correspond to regions deleted in the Ku86 cDNAs of mutants XR-V9B and XR-V15B.

correlated with the level of DEB activity observed, suggesting that Ku was limiting in abundance for all these processes.

Nucleotide sequence of the Chinese hamster Ku86 cDNA. To study the molecular nature of the defects in XR-V15B and XR-V9B, another X-ray-sensitive Chinese hamster mutant belonging to complementation group 5 (53, 54), the cDNA of Ku86 from parental cell line V79B was sequenced. Total V79B RNA was used to synthesize oligo(dT)-primed cDNA by murine leukemia virus reverse transcriptase. Subsequently, this cDNA was amplified by PCR with Ku86-specific primers Ku-5 and Ku-3b, which were based on the sequence of Syrian hamster Ku86 cDNA (Fig. 4). By using a series of primer walking steps, the resulting 2.3-kb PCR fragment containing the entire open reading frame of Ku86 was sequenced.

The nucleotide and predicted amino acid sequences of the Chinese hamster cDNA of Ku86 are presented in Fig. 5. The Chinese hamster Ku86 cDNA sequence is 95.5, 87.4, and 80.3% identical to the Syrian hamster (17), mouse (10), and human (32, 50) homologs, respectively (Fig. 6).

Sequence analysis of Ku86 cDNAs from XR-V15B and XR-V9B mutants. To determine whether the Ku86 cDNAs in the X-ray-sensitive mutants XR-V15B and XR-V9B were abnormal, PCR-amplified Ku86 cDNA was sequenced as described in Materials and Methods. Comparison of these sequences with the wild-type Ku86 sequence revealed a deletion of 138 bp from nucleotides +1114 through +1251 in the XR-V15B cDNA and a deletion of 252 bp from +799 through +1050 in

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-30  aag taa cca aac cgc ccg tgg acc agc aac ATG GCG TGG TCC GCT AAT AAG GCA GCT GTT GTG CTG
  1                                        M  A  W  S  A  N  K  A  A  V  V  L
 37  TGT ATG GAT GTG GGC GTT GCT ATG GGT AAC TCT TTT CCT GGC GAA GAA TCC TCG TTT GAA CAA GCA
 13   C  M  D  V  G  V  A  M  G  N  S  F  P  G  E  E  S  S  F  E  Q  A
103  AAG AAA GTG ATG ACT ATG TTT GTC CAA CGA CAG GTG TTT TCT GAG AGC AAG GAT GAG ATT GCC TTG
 35   K  K  V  M  T  M  F  V  Q  R  Q  V  F  S  E  S  K  D  E  I  A  L
169  GTC CTC TTT GGA ACA GAC AAC ACT AAC AAC GCC CTT GCT AGT GAG GAC CAG TAT CAG AAC ATC ACA
 57   V  L  F  G  T  D  N  T  N  N  A  L  A  S  E  D  Q  Y  Q  N  I  T
235  GTG CAC AGA CAC CTG ATG CTA CCA GAT TTT GAT TTG CTG GAA GAC ATC GAA AGC AAA ATC CAA CTA
 79   V  H  R  H  L  M  L  P  D  F  D  L  L  E  D  I  E  S  K  I  Q  L
301  GGC TCT CGA CAA GCT GAC ATC TTG GAC GCC CTG ATT GTG TGC ATG GAT TTG ATT CAG CGT GAA ACC
101   G  S  R  Q  A  D  I  L  D  A  L  I  V  C  M  D  L  I  Q  R  E  T
367  ATA GGG AAG AAG TTT GAG AAG AAG CAT ATT GAA GTG TTC ACT GAC CTC AGC AGC CCA TTC AGC CAA
123   I  G  K  K  F  E  K  K  H  I  E  V  F  T  D  L  S  S  P  F  S  Q
433  GAT CAA CTG GAT GTT ATC ATT TGT AAC TTG AAG AAG TCT GGC ATC TCC CTG CAG TTC TTC CTG CCT
145   D  Q  L  D  V  I  I  C  N  L  K  K  S  G  I  S  L  Q  F  F  L  P
499  TTT CCA ATC AGC AAG AAC GAT GAG ACT GGG GAC AGA GGA GAT GGT GAC TTG GGC TTG GAC CAC TGT
167   F  P  I  S  K  N  D  E  T  G  D  R  G  D  G  D  L  G  L  D  H  C
565  GGA CCC TCC TTT CCT CAA AAA GGA ATT ACT GAG CAG CAA AAG GAA GGC ATC TGC ATG GTG GAG AGG
189   G  P  S  F  P  Q  K  G  I  T  E  Q  Q  K  E  G  I  C  M  V  E  R
631  GTG ATG GTG TCT TTA GAA GGT GAA GAT GGG CTG GAT GAG ATC TAC TCC TTC AGT GAG AGT CTA CGG
211   V  M  V  S  L  E  G  E  D  G  L  D  E  I  Y  S  F  S  E  S  L  R
697  CGG CTG TGT GTC TTT AAG AAG ATT GAG AGG CGC TCC ATG CCC TGG TCC TGC CAG CTG ACC ATC GGC
233   R  L  C  V  F  K  K  I  E  R  R  S  M  P  W  S  C  Q  L  T  I  G
763  CCT GAT TTG TCT ATA AAG ATT GTA GCC TAT AAA TCG ATC GTA CAG GAG AAA GTT AAA AAG AGC TGG
255   P  D  L  S  I  K  I  V  A  Y  K  S  I  V  Q  E  K  V  K  K  S  W
829  ATA GTT GTG GAT GCA AGA ACT CTA AAG AAG GAA GAT ATA AGA AAA GAA ACT GTC TAT TGC TTA AAC
277   I  V  V  D  A  R  T  L  K  K  E  D  I  R  K  E  T  V  Y  C  L  N
895  GAT GAT GAC GAA ACT GAA GTT TCC AAA GAG GAC ACT ATT CAA GGG TTC CGC TAC GGA AGT GAT ATA
299   D  D  D  E  T  E  V  S  K  E  D  T  I  Q  G  F  R  Y  G  S  D  I
961  ATT CCT TTT TCT AAA GTG GAT GAG GAA CAA ATG AAA TAT AAA TCA GAG GGG AAG TGC TTC TCT GTT
321   I  P  F  S  K  V  D  E  E  Q  M  K  Y  K  S  E  G  K  C  F  S  V
1027 TTG GGA TTC TGT AGA TCT TCT CAG GTC CAC AGG AGA TTC TTT ATG GGA TAT CAA GTT CTG AAG GTC
343   L  G  F  C  R  S  S  Q  V  H  R  R  F  F  M  G  Y  Q  V  L  K  V
1093 TTT GCA GCA AAA GAT GAT GAG GCA GCG GCT GTT GCT CTC TCT TCC CTT ATT CAT GCT TTG GAT GAA
365   F  A  A  K  D  D  E  A  A  A  V  A  L  S  S  L  I  H  A  L  D  E
1159 TTA AAC ATG GTC GCC ATC GTT CGA TAT GCT TAT GAC AAA AGA GCT AAT CCT CAA GTT GGT GTA GCC
387   L  N  M  V  A  I  V  R  Y  A  Y  D  K  R  A  N  P  Q  V  G  V  A
1225 TTT CCT TAT ATT AAG GAT TCC TAT GAG TGT TTA GTT TAT GTG CAG CTG CCT TTC ATG GAA GAC TTG
409   F  P  Y  I  K  D  S  Y  E  C  L  V  Y  V  Q  L  P  F  M  E  D  L
1291 CGG CAA TAC ATG TTT TCA TCA CTG AAA AAC AAT AAG AAA TGC ACT CCC ACA GAG GCA CAG TTG AGT
431   R  Q  Y  M  F  S  S  L  K  N  N  K  K  C  T  P  T  E  A  Q  L  S
1357 GCT ATT GAT GAT CTG ATT GAG TCT ATG AGC TTG GTA AAG AAA AGC GAG GAA GAA GAT ACC ATT GAA
453   A  I  D  D  L  I  E  S  M  S  L  V  K  K  S  E  E  E  D  T  I  E
1423 GAC TTG TTT CCA ACC TCC AAA ATT CCA AAT CCC GAA TTT CAG AGA TTT TTC CAG TGT CTG CTG CAC
475   D  L  F  P  T  S  K  I  P  N  P  E  F  Q  R  F  F  Q  C  L  L  H
1489 AGA GTC TTA CAT CCC CAA GAG CGT TTG CCC CCA ATT CAG CAG CAC ATT TTG AAT ATG CTG AAT CTC
497   R  V  L  H  P  Q  E  R  L  P  P  I  Q  Q  H  I  L  N  M  L  N  L
1555 CCC ACT GAG ATG AAA GCC AAA TGT GAG ATT CCT CTC TCT AAA GTG AGG ACC CTT TTC CCG CTG ACA
519   P  T  E  M  K  A  K  C  E  I  P  L  S  K  V  R  T  L  F  P  L  T
1621 GAA GCC GTC AAG AAA AAG GAC CAA GTG ACT GCT CAG GAC ATT TTC CAA GAC ATT CAC GAA GAG GGG
541   E  A  V  K  K  K  D  Q  V  T  A  Q  D  I  F  Q  D  I  H  E  E  G
1687 CCC GCT GCC AAA AAA TGT AAG ACA GAG AAA GAA GAG GGT CAC ATC AGT ATC TCT AGC GTG GCT GAA
563   P  A  A  K  K  C  K  T  E  K  E  E  G  H  I  S  I  S  S  V  A  E
1753 GGG AAT GTC ACC AAG GTT GGA AGT GTG AAT CCT GTT GAA AGC TTC CGT GTT CTA GTG AGG CAG AAG
585   G  N  V  T  K  V  G  S  V  N  P  V  E  S  F  R  V  L  V  R  Q  K
1819 ATT GCC AGC TTT GAG CAG GCA AGT CTC CAG CTA ATA AGT CAC ATT GAG CAG TTT TTG GAT ACC AAC
607   I  A  S  F  E  Q  A  S  L  Q  L  I  S  H  I  E  Q  F  L  D  T  N
1885 GAA ACG CTG TAT TTT ATG AAG AGT ATG GAG TGC ATC AAA GCT TTC CGG GAG GAG GCC ATC CAG TTT
629   E  T  L  Y  F  M  K  S  M  E  C  I  K  A  F  R  E  E  A  I  Q  F
1951 TCA GAA GAG CAG CGC TTC AAC AGC TTC CTG GAA GCC CTT CGA GAG AAA GTG GAA ATT AAG CAA TTA
651   S  E  E  Q  R  F  N  S  F  L  E  A  L  R  E  K  V  E  I  K  Q  L
2017 AAT CAT TTC TGG GAA ATT GTT GTT CAG GAT GGA GTT ACT CTG ATT ACC AAG GAT GAA GGC TCT GGA
673   N  H  F  W  E  I  V  V  Q  D  G  V  T  L  I  T  K  D  E  G  S  G
2083 AGC TCT GTC ACA ACT GAG GAA GCC ACA AAG TTC CTG GCT CCC AAA GAC AAA GCA AAA GAA GAT GCA
695   S  S  V  T  T  E  E  A  T  K  F  L  A  P  K  D  K  A  K  E  D  A
2149 GCA GGA CTT GAA GAA GGT GGC GAT GTG GAT GAT TTA CTG GAT ATG ATA TAG acc atg gat gtg ttg
717   A  G  L  E  E  G  G  D  V  D  D  L  L  D  M  I  *
2215 gga atc taa gca tgc cat ctc cgt tgc tga gag ctc aga aag agc atc ctg gaa gcc at
```

FIG. 5. Nucleic acid and predicted amino acid sequences of Chinese hamster Ku86 cDNA. The deleted nucleotides in XR-V9B and XR-V15B are underlined. Lowercase letters, untranslated nucleotides; asterisk, stop codon.

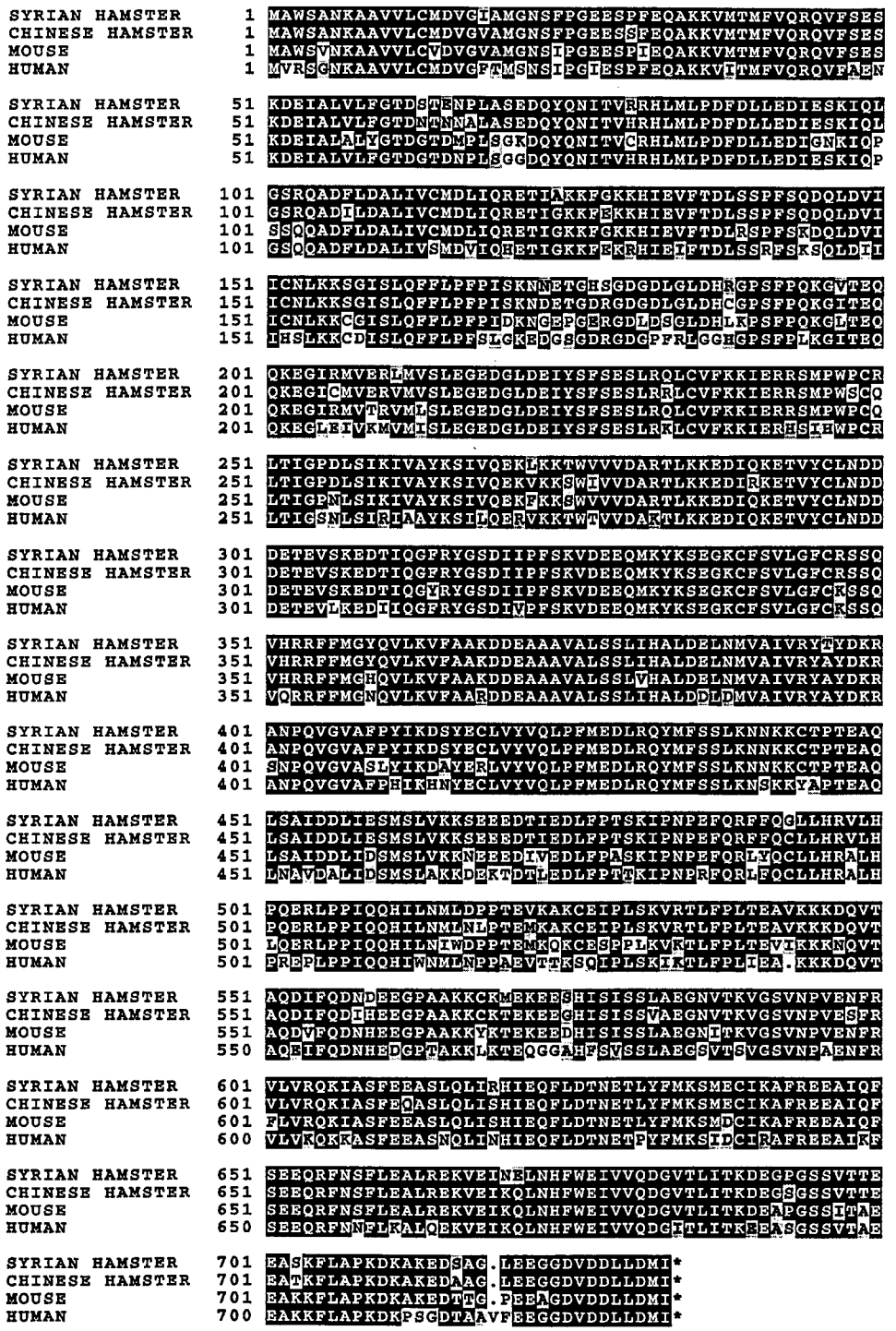

FIG. 6. Amino acid alignment of Syrian and Chinese hamster, mouse, and human Ku86 proteins. Alignment was determined by using the Boxshade program (8a). Identical amino acids are highlighted in black. Functionally conserved amino acids are highlighted in gray. Conserved amino acids are classified as follows: V, I, L, and M; D, E, Q, and N; F, Y, and W; G, S, T, P, and A; and K, R, and H.

the XR-V9B cDNA (Fig. 6). These deletions do not shift the reading frame and correspond to a deletion of 46 amino acid residues from codons 372 through 417 in XR-V15B and to a deletion of 84 codons from codons 267 through 350 in XR-V9B (Fig. 6). No additional mutations in these Ku86 cDNAs could be detected in either mutant cell line.

To eliminate the possibility that the deletions in XR-V15B and XR-V9B were cloning artifacts, RT-PCR was performed with primers adjacent to these deletions. Primers Ku-12 and Ku-13 were designed to generate a 448-bp fragment spanning the deletion in the XR-V15B mutant, and primers Ku-5 and Ku-8b were used to generate a 1,246-bp fragment spanning the deletion in XR-V9B. RT-PCR showed that the XR-V15B mutant contained a PCR fragment consistent with a length of 310

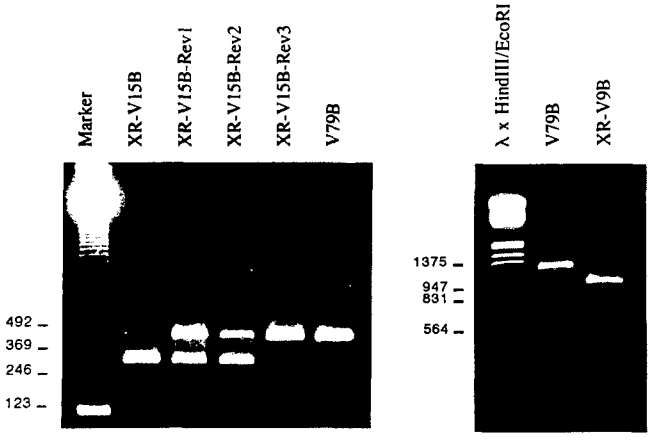

FIG. 7. RT-PCR of XR-V15B, XR-V15B revertants, and V79B with primers Ku-12 and Ku-13 (left) and of V79B and XR-V9B with primers Ku-5 and Ku-8b. The positions of molecular size markers (in base pairs) are shown on the left of each panel.

bp, instead of the wild-type fragment of 448 bp (Fig. 7). Similarly, XR-V9B contains a PCR fragment consistent with a length of 994 bp, instead of the wild-type 1,246-bp fragment (Fig. 7). Thus, we observed PCR products shorter than the wild-type product, confirming the deletions in the Ku86 cDNAs in both mutants.

RT-PCR analysis of X-ray-resistant revertants of XR-V15B. To examine the mechanism responsible for the reversion of the X-ray sensitivity in XR-V15B, we analyzed three X-ray-induced X-ray-resistant revertant cell clones, XR-V15B-Rev1, XR-V15B-Rev2, and XR-V15B-Rev3 (see Materials and Methods). All three revertants showed wild-type levels of X-ray resistance (data not shown), suggesting that Ku86 function was completely restored in each case. To determine the status of the deleted cDNA in these revertants, RT-PCR was performed with primers Ku-12 and Ku-13. Revertants XR-V15B-Rev1 and XR-V15B-Rev2 showed a normal, wild-type fragment of 448 bp, as well as a smaller fragment of 310 bp (Fig. 7). Thus, we concluded that the XR-V15B-Rev1 and XR-V15B-Rev2 cell lines expressed one mutant and one wild-type copy of Ku86. In contrast, XR-V15B-Rev3 expressed only a wild-type fragment of 448 bp (Fig. 7). This indicated that an event leading to the disappearance of the deletion occurred in XR-V15B-Rev3.

DISCUSSION

The data presented in this paper demonstrate that defects in the *Ku86* gene are responsible for the phenotypes of complementation group 5 hamster mutants. First, transfection of XR-V15B cells with a cDNA encoding the hamster *Ku86* gene was capable of fully restoring DEB activity, Ku70 protein levels, X-ray resistance, and V(D)J recombination. Second, XR-V15B and XR-V9B cells express mRNAs which encode Ku86 proteins containing significant in-frame deletions. This is the first direct evidence that mutant cells in group 5 contain mutations in the *Ku86* gene. Thus, the *XRCC5* gene defective in group 5 cells is identical to the *Ku86* gene.

Rescue of group 5 mutant cells with hamster Ku86 cDNA. The expression of Syrian hamster Ku86 cDNA in XR-V15B Chinese hamster mutant cells was capable of restoring DEB activity to levels comparable to those of the wild-type V79 cell line. The Ku70 protein levels in the clone expressing the highest level of DEB activity increased to wild-type levels (Fig. 1, lane 5). The restoration of Ku70 levels in mutant cells by hamster Ku86 suggests that a functional Ku86 protein is necessary for stabilization of the Ku70 polypeptide. Conversely, there is evidence that functional Ku70 protein is required for stabilization of Ku86 (39). Thus, it appears that if Ku70 and Ku86 are not complexed to each other, they are degraded in the cell.

Our results suggest that Ku levels are limiting in the processes of DNA repair and V(D)J recombination. Although clones expressing wild-type levels of DEB activity showed complete restoration of X-ray resistance and V(D)J recombination activity, clones expressing less than wild-type levels of DEB activity showed only partial rescue of X-ray resistance and V(D)J recombination. Between 16 and 40 DNA DSBs are induced in mammalian cells by 1 Gy of ionizing radiation (11). Therefore, even the highest dose used in our experiments, 6 Gy, induced at most 240 DSBs per cell. Each wild-type human cell contains about 400,000 molecules of Ku (30). Therefore, we were surprised to find that transfected cells expressing Ku at levels only modestly less than wild-type levels were partially impaired in both X-ray resistance and V(D)J recombination.

There are several possible explanations. Multiple Ku molecules might be necessary to process each end of a broken DNA molecule. Although Ku binds initially to DNA ends, it can translocate inward along the DNA molecule either in an ATP-independent manner or as an ATP-dependent helicase (9, 48). Whether these functions are required for the repair of DNA DSBs remains unknown. Alternatively, Ku might be localized to subcellular compartments that limit its access to DNA strand breaks. In fact, Ku has been reported to change nuclear localization in a cell cycle-dependent way (49). Additionally, Ku is known to be posttranslationally modified (5, 33); it is possible that only one form of Ku is able to function in DNA repair. Thus, in cells expressing low levels of Ku, the functional form of Ku may be present at levels too low to completely restore DNA repair. Finally, it is possible that Syrian hamster Ku86, whose amino acid sequence is 4.5% diverged from Chinese hamster Ku86, rescues Chinese hamster mutants less efficiently so that Ku levels only appear to be limiting. To rule out this possibility, we are currently transfecting the Chinese hamster Ku86 clone into Chinese hamster mutant cells.

Defects in the mRNA from mutant cells in group 5. The deletions in mRNAs recovered from XR-V15B and XR-V9B cells do not shift the open reading frame but instead remove 46 and 84 amino acids from two different regions within the encoded Ku86 protein. The deletion of either region leads to an apparent failure to stabilize Ku70 protein, the loss of DEB activity, and the concomitant impairment of X-ray resistance and V(D)J recombination in the cells. Therefore, the deleted domains appear to play an essential role in the function of the Ku86 protein.

Chinese hamster cell lines have been the source of a broad spectrum of phenotypically recessive mutants corresponding to a large number of different genetic loci (8, 41). Normally, recessive mutations should not be found at such high frequencies in diploid cells. Siminovitch (41) has suggested that a substantial part of the genome in Chinese hamster cell lines is effectively haploid so that many genes are represented by only one functional allele. Functional hemizygosity can arise from the silencing of genes by methylation, and group 5 hamster cell mutants might have two alleles of the *XRCC5* gene, with one allele inactivated by mutation and another wild-type allele silenced by methylation (22). RNAs derived from revertants XR-V15B-Rev1 and XR-V15B-Rev2 harbor both wild-type and deleted Ku86 sequences. Thus, a wild-type *Ku86* gene was activated in these revertants. Consistent with this observation,

we have previously shown that azacytidine, an agent which induces demethylation of silenced genes, greatly increases the frequency of revertants in XR-V15B (36, 37). Therefore, the mechanism for activating the *Ku86* gene in these revertants appears to be via demethylation of a silenced allele.

The third revertant, XR-V15B-Rev3, contained only wild-type Ku86 mRNA, suggesting that demethylation was not involved in reactivation of this gene. The deleted mRNA region may have reappeared in this revertant by several mechanisms: (i) homologous recombination between the active mutant allele and the inactive normal allele; (ii) gene conversion of the active allele, with the inactive allele as a template; or (iii) reversion of the mutation in the active (defective) allele. The third possibility suggests that the deletion in the Ku86 mRNA is the result of defective splicing caused by a revertible mutation at a donor or acceptor splice site in the gene. Significantly, the XR-V15B mutant was induced by ethylnitrosourea, which almost exclusively induces point mutations (40), so the molecular defect in XR-V15B might be a point mutation in the genomic DNA which leads to defective splicing of the Ku86 mRNA. In support of this possibility, the 3′ end of each deletion contains a short pyrimidine-rich tract followed by an AG dinucleotide, which is similar to the consensus mRNA splice acceptor sequence and thus could act as an acceptor for defective splicing (Fig. 5). However, confirmation of such mutations will require the sequencing of genomic DNA.

In summary, the rescue of XR-V15B cells by a Ku86 cDNA and the identification of defects in the cDNAs encoding Ku86 in XR-V15B and XR-V9B cells demonstrate that (i) the defect in complementation group 5 mutant cells must be in the *Ku86* gene, (ii) Ku86 is essential for DSB repair and V(D)J recombination, and (iii) Ku86 expression is required for the accumulation of Ku70 protein.

ACKNOWLEDGMENTS

A.E. and V.S. contributed equally to this work.

We thank Visakha Goonewardena for assistance; Rachel Gerstein, Greg Donoho, Albert Pastink, and Harry Vrieling for advice; and Byung Joon Hwang for helpful discussions.

This work was supported in part by grant DAMD17-94-J-4350 to G.C. from the U.S. Department of the Army, by grant AI35763 from the National Institutes of Health to E.A.H., and by grant 9.0.6 to M.Z. from the J. A. Cohen Institute, Interuniversity Research Institute for Radiopathology and Radiation Protection. E.A.H. is a Leukemia Society of America Scholar. W.K.R. is a Howard Hughes Medical Institute predoctoral scholar.

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[CANCER RESEARCH 57, ●●●–●●●, January 1, 1997]

DNA-dependent Protein Kinase Is Not Required for Accumulation of p53 or Cell Cycle Arrest after DNA Damage[1]

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ABSTRACT

In response to DNA damage, cells transduce a signal that leads to accumulation and activation of p53 protein, transcriptional induction of several genes, including *p21*, *gadd45*, and *gadd153*, and cell cycle arrest. One hypothesis is that the signal is mediated by DNA-dependent protein kinase (DNA-PK), which consists of a catalytic subunit (DNA-PK_{cs}) and a regulatory subunit (Ku). DNA-PK has several characteristics that support this hypothesis: Ku binds to DNA damaged by nicks or double-strand breaks, DNA-PK_{cs} is activated when Ku binds to DNA, DNA-PK will phosphorylate p53 and other cell cycle regulatory proteins *in vitro*, and *DNA-PK_{cs}* shares homology with *ATM*, which is mutated in ataxia telangiectasia and involved in signaling the p53 response to ionizing radiation. The hypothesis was tested by analyzing early passage fibroblasts from scid mice, which are deficient in DNA-PK. After exposure to ionizing radiation, UV radiation, or methyl methanesulfonate, severe combined immunodeficient and wild-type cells were indistinguishable in their response. The accumulation of p53, induction of *p21*, *gadd45*, and *gadd153*, and arrest of the cell cycle in G_1 and G_2 occurred normally. Therefore, DNA-PK is not required for the p53 response or cell cycle arrest after DNA damage.

INTRODUCTION

When cells are exposed to DNA-damaging agents, they initiate a complex response that includes the arrest of cell cycle progression until the damage is repaired (1, 2). Agents that induce this response include IR,[3] UV radiation, and chemical agents such as MMS. IR induces DNA nicks, double-strand breaks, and base damage; UV radiation induces cyclobutane pyrimidine dimers, 6–4 photoproducts, and other lesions, including DNA nicks acquired during excision repair of photoproducts; and MMS alkylates DNA, leading to spontaneous and enzymatic depurinations and DNA nicks from excision repair of abasic sites.

Several components of the pathways that arrest the cell cycle in response to DNA damage have now been identified in mammalian cells. One component of the G_1 checkpoint is p53, a M_r 53,000 protein that is capable of binding to specific DNA sequences as a transcriptional activator and is normally present in the cell at very low levels. When cells are exposed to DNA damage, p53 undergoes posttranslational modification that stabilizes the protein and contributes to its accumulation (3–5). Cells lacking p53 fail to undergo G_1 arrest following exposure to DNA-damaging agents (6, 7). The *p53* gene is mutated in 50% of all human cancers, which suggests that the p53-dependent G_1 checkpoint pathway is critical for suppressing carcinogenesis.

The *p21* gene (also named *Cip1*,*Waf1*, *Cap20*, or *Sdi1*) includes a p53 DNA-binding site in its promoter region and is transcriptionally activated by p53 (8). Thus, *p21* is induced by DNA damage and p21 protein interacts directly with cyclin-dependent kinases, inhibiting their activity and arresting the cell cycle (6, 9, 10).

Several genes are induced by growth arrest and DNA damage (*gadd*) and include the *gadd45* and *gadd153* genes (11). Like *p21*, the *gadd45* promoter contains a p53-binding site, and *gadd45* transcription is induced by IR via a p53-dependent pathway (12). The *gadd45* gene is also induced by UV radiation and MMS by a p53-independent pathway. The *gadd153* gene is induced by UV radiation and MMS by a p53-independent pathway and is not induced by IR (13).

Despite progress in identifying many of the damage-response genes, the molecule that detects DNA damage and initiates the signal for the subsequent cellular response remains a mystery. One candidate molecule is DNA-PK, a serine/threonine protein kinase composed of a catalytic subunit (DNA-PK_{cs}) and a regulatory subunit (Ku; Ref. 14). The catalytic subunit DNA-PK_{cs} is a M_r 465,000 polypeptide that is normally inactive, but is activated by the binding of Ku to one of its DNA substrates. Ku was identified as an autoantigen in several autoimmune diseases; it is a heterodimer of M_r 70,000 and 86,000 (Ku70 and Ku86) that binds to DNA ends, nicks, gaps, and stem-loop structures (15, 16). These Ku substrates are noteworthy since DNA ends are induced by IR, and DNA nicks are produced as an intermediate during excision repair of UV- and MMS-induced damage. Such strand breaks appear to trigger the p53-dependent damage response (17).

DNA-PK can phosphorylate a number of proteins *in vitro*, including Ku, p53, and other proteins with roles in replication or regulation of the cell cycle: RPA, c-myc, c-fos, c-jun, and topoisomerases I and II (18). DNA-PK appears to be required for the phosphorylation of Ku and RPA *in vivo* (19). Furthermore, the phosphorylation of p53 by DNA-PK might be physiologically significant: DNA-PK phosphorylates p53 *in vitro* at serines 15 and 37 in the amino terminal transactivation domain of p53, and site-directed mutagenesis of serine 15 leads to stabilization of p53, suggesting that phosphorylation of that site might affect the lifetime of p53 (20).

The kinase domain of DNA-PK_{cs} shares homology with the kinase domain from the gene (*ATM*) that is mutated in ataxia telangiectasia (21). Mutations in the *ATM* gene lead to IR hypersensitivity, reduced ability to arrest DNA synthesis and mitosis, and a delayed p53 response after IR. Thus, the *ATM* gene acts upstream of p53 in the G_1 checkpoint pathway (4, 6, 22), raising the possibility that DNA-PK might also act in a checkpoint pathway.

DNA-PK has an established role in detecting and repairing DNA double-strand breaks. Mutant rodent cells in three X-ray-sensitive complementation groups (groups 4, 5, and 7) are defective in repairing the double-strand breaks induced either by IR or by V(D)J recombination (23, 24). Group 5 cells lack Ku DNA end-binding activity (25, 26), are rescued by transfection of *Ku86* cDNA (27, 28), and have mutations in the *Ku86* gene (29). Group 7 cells contain Ku DNA end-binding activity, but contain severely decreased levels of immunoreactive and enzymatically active DNA-PK_{cs} protein (30–32). Furthermore, group 7 cells are rescued by centromeric fragments of

Received 5/30/96; accepted 11/1/96.

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[1] Supported by U.S. Army Medical Research and Material Command DAMD 17–94-J-4350 (G. C.), USPHS Grant NIH CA42765 (W. K. K.), and a predoctoral fellowship from the Howard Hughes Medical Institute (W. K. R.).

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[3] The abbreviations used are: IR, ionizing radiation; DNA-PK, DNA-dependent protein kinase; DNA-PK_{cs}, DNA-dependent protein kinase catalytic subunit; MMS, methyl methanesulfonate; scid, severe combined immunodeficient; RPA, replication protein A; BrdUrd, bromodeoxyuridine; NHF, normal human fibroblast.

human chromosomal DNA containing the *DNA-PK$_{cs}$* gene (30–32). Group 7 includes cells from the scid mouse, which is hypersensitive to IR and lacks mature B and T cells due to a deficiency in V(D)J recombination (33).

DNA-PK has several hallmarks that would be expected for a molecule that couples DNA damage to arrest of the cell cycle. First, DNA-PK binds to DNA damaged by nicks or double-strand breaks and already has an established role in recognizing and repairing DNA damage in intact cells. Second, its kinase activity is quiescent under normal conditions but is activated by DNA damage. Third, potential targets for DNA-PK include p53 and other proteins that play roles in regulating the cell cycle. Fourth, the homology of DNA-PK$_{cs}$ to ATM suggests that DNA-PK$_{cs}$ might play a similar role in regulating the cell cycle after DNA damage.

In this article, we test the hypothesis that DNA-PK is essential for transducing DNA damage into the subsequent cellular responses. Since established cell lines often have abnormal regulation of their cell cycles, studies were performed on early passage fibroblasts derived from newborn scid mice, which are defective in DNA-PK. scid and wild-type cells were exposed to DNA damage and compared for accumulation of p53 protein, induction of the *p21*, *gadd45*, and *gadd153* genes, and G_1 and G_2 checkpoint responses.

MATERIALS AND METHODS

Cell Culture. Early passage embryo fibroblast cultures derived from p53 deficient (−/−) mice were generously provided by L. Donehower (34). The p53−/− cells were grown in DMEM supplemented with 10% FCS. Early passage skin fibroblast cultures were generated from newborn scid (33) and BALB/c mice generously provided by I. Weissman. scid mice were originally derived from the C.B-17 mouse lineage which differs from BALB/c exclusively at the immunoglobulin heavy chain locus *IgH* (33). Agouti wild type mice were generously provided by G. Barsh. Within 3–8 h after birth, newborn mice were sacrificed and the dorsal and ventral skins were surgically removed. Individual skins were washed repeatedly with PBS, and all traces of blood and connective tissue were removed. The skins were then placed in 10 ml of trypsin-EDTA (0.05% trypsin and 0.53 mM EDTA), which had been prewarmed to 37°C where they were dispersed by extensive cutting and vigorous pipetting. The culture was incubated at 37°C for 30 min, 5 ml of fresh warm trypsin-EDTA were added, and the culture was incubated further at 37°C for 15 min. The suspension was transferred to a 50-ml conical tube, and the remaining large fragments were allowed to settle to the bottom. The supernatant was carefully removed, and the cells were pelleted by spinning for 5 min at 1000 rpm. The cells were then resuspended in DMEM supplemented with 10% FCS and plated onto 10-cm tissue culture dishes. All cultures were maintained at 37°C in 5% CO_2. Newborn human fibroblasts (NHF1) were prepared and maintained as described previously (6, 35).

For treatments with DNA-damaging agents, 2×10^6 cells were plated onto 10-cm tissue culture dishes 24 h before treatment. For UV treatment, cells were rinsed with PBS and exposed, uncovered, to UV radiation from a germicidal lamp at a flux of 1 J/m^2/s. Following treatment, fresh medium was added to the plates for the remaining incubation. For MMS treatment, cells were placed in fresh medium containing 100 μg/ml MMS and incubated at 37°C for the duration of the experiment. For IR treatment, cells were exposed to a ^{137}Cs source with a flux of 11.5 Gy/min. To test IR-sensitivity of the primary fibroblast cultures, 3×10^4 scid or BALB/c cells were plated in duplicate and treated with 0, 2.5, 5, or 10 Gy. Five days after treatment, the remaining cells were trypsinized and counted.

Western Blot Analysis. Cells were lysed in a SDS-containing buffer [0.125 M Tris-Cl (pH 6.8), 4% SDS, 20% glycerol, 0.008% bromphenol blue, and 50 mM DTT] at a concentration of 10^7 cells/ml, immediately boiled for 10 min, and loaded directly onto the gel or stored at −80°C. Samples (2×10^5 cell equivalents/lane) were resolved by a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose GSWP membrane (Millipore, Bedford, MA), and probed with monoclonal p53 antibody 240 or M19 (Santa Cruz Biotechnology, Santa Cruz, CA) followed by horseradish peroxidase-conjugated goat anti-mouse IgG or swine anti-goat IgG, respectively (Bio-Rad, Richmond, CA). Antibody binding was detected using enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

Northern Blot Analysis. Total RNA was prepared using RNA STAT-60 reagent (Tel-Test "B, Friendswood, TX) according to the manufacturer's instructions. Probes used for Northern blot analysis included *Cip1* cDNA (from S. Elledge), *gadd45* and *gadd153* cDNA (from A. Fornace), and human β-actin cDNA (from P. Berg). They were labeled with [^{32}P]dCTP by random hexamer labeling. Total RNA (10 μg) was denatured and run on a 0.8% formaldehyde agarose gel. The RNA was transferred to a Hybond nylon filter (Amersham Corp.) and cross-linked with Stratalinker (Stratagene, La Jolla, CA). The filter was hybridized to ^{32}P-labeled probes at 65°C for 24 h in 1× Nylon wash (14% SDS, 130 mM Na_2HPO_4, 14 mM EDTA, and 0.2% Triton X-100). The filter was washed sequentially in 0.5×, 0.25×, and 0.1× Nylon wash for 30 min each at 65°C. The filter was then autoradiographed on XAR-5 X-ray film at −80°C. To reprobe the filter, the filter was stripped by washing in 0.03× Nylon wash for 2 or more h at 65°C and then hybridized to the new ^{32}P-labeled probe.

Cell Cycle Checkpoint Response. Fibroblast cultures were assayed for G_1 arrest by incubating asynchronously proliferating cells with 10 μM BrdUrd (Sigma Chemical Co., St. Louis, MO) for 2 h, beginning 6–8 h after mock treatment or exposure to IR. BrdUrd-labeled nuclei were quantified by flow cytometry after incubation with FITC-labeled anti-BrdUrd antibody (Becton Dickinson, Bedford, MA) as described previously (35). G_2 delay was assayed as the fraction of cells in mitosis at various times after exposure of asynchronously proliferating cells to IR as described previously (35, 36). To determine the mitotic fraction, mitotic figures were counted in 50–200 consecutive high-power fields. Enough fields were viewed to ensure that at least 20 mitotic cells were counted in the sham control.

RESULTS

Preliminary Experiments on Immortalized Cell Lines. Our initial strategy in testing the possibility that DNA-PK might be involved in signaling DNA damage-mediated cell cycle regulation was to utilize immortalized mouse and hamster cell lines containing defects in the individual components of DNA-PK. Western blot analysis of p53 protein levels in wild-type and mutant immortalized hamster cells revealed high levels of constitutive p53 expression, which was not induced significantly upon treatment with IR or UV (data not shown). In immortalized wild-type (C.B-17) and scid (SF-7) mouse cells, the constitutive expression level of p53 was also abnormally high (data not shown). In nonimmortalized primary cells, the basal expression of p53 is extremely low due to the short half-life of p53 protein (3, 37). Therefore, we chose to pursue these studies in early passage fibroblasts.

Phenotype of Early Passage Fibroblast Cells. Early passage fibroblast cultures from newborn scid and wild-type (agouti and BALB/c) mice were tested for two relevant phenotypes. The early passage scid cells contained normal levels of Ku DNA end-binding activity (data not shown) as was found for immortalized scid cells using methods described previously (26). In addition, the cells were tested for sensitivity to IR. The scid cells were approximately 10-fold more sensitive than wild-type cells at a dose of 10 Gy (Fig. 1), which was the same level of sensitivity as previously measured for immortalized scid cell lines (38, 39).

DNA Damage-induced Accumulation of p53. The accumulation of p53 in response to UV radiation was determined by measuring p53 protein levels over time after exposing cells to UV radiation (Fig. 2). The p53 accumulation in wild-type cells followed a prolonged time course from 4 h to at least 12 h, as reported previously (22). The p53 accumulation in scid cells was not significantly different from that in wild-type cells in four independent experiments with two different antibodies (M19 and 240).

The accumulation of p53 after IR was determined in early passage scid cells (Fig. 3). IR at doses of 2–20 Gy has been reported to

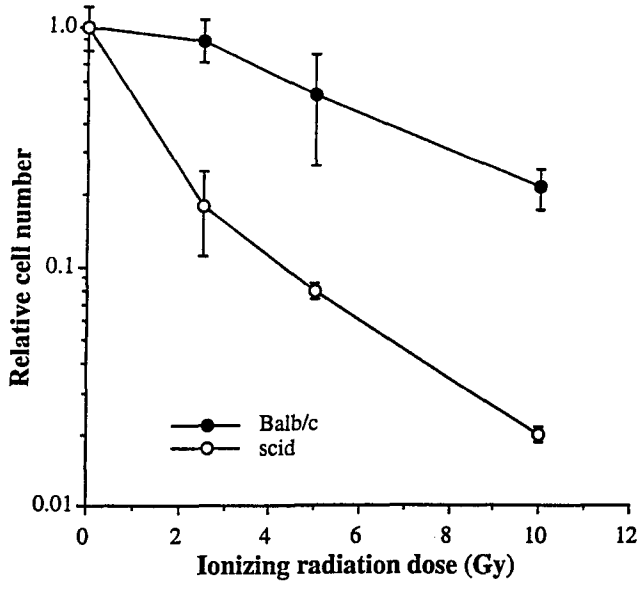

Fig. 1. scid cells are hypersensitive to IR. Early passage fibroblasts from wild-type BALB/c and scid mice were treated with 0, 2.5, 5, and 10 Gy IR. After 5 days of growth in culture, the surviving cells were trypsinized and counted. *Bars*, SD.

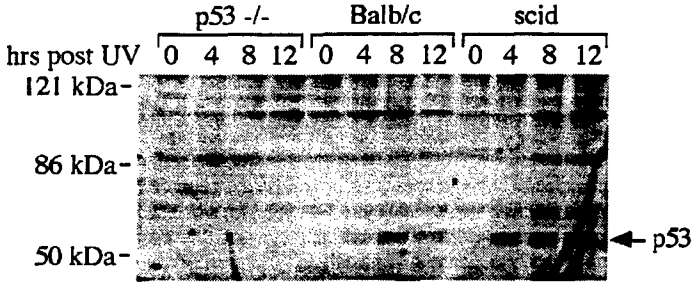

Fig. 2. scid cells accumulate normal levels of p53 after UV damage. Early passage fibroblasts from p53 knockout mice (p53−/−), wild-type BALB/c mice, and scid mice were treated with 40 J/m^2 UV radiation. Cell lysates were harvested after 0, 4, 8, and 12 h, and the accumulation of p53 was detected by Western blot using p53 antibody M19 (see "Materials and Methods"). Each lane was loaded with lysate from 2 × 10^5 cells. The position of the size markers is indicated to the *left*, and the band corresponding to p53 is indicated with an *arrow*. Note that this band, but not other background bands, is absent in p53−/− cell lysates. The slight differences between scid and wild-type cells in this particular blot were not significant when the differences in intensities of the nearby nonspecific cross-reacting bands at M_r 60,000 and 90,000 were taken into account. The regional variation in staining may have been caused by variation in protein transfer, antibody binding, or the chemiluminescence reaction.

increase p53 levels, with a brief peak (approximately 2–5-fold) 1–2 h after irradiation (5, 7). Therefore, wild-type, scid, and p53−/− cells were analyzed with Western blot for p53 protein expression 1, 2, and 4 h after treatment with 10 Gy IR. Wild-type and scid cell lines showed no significant difference in the p53 induction profile over a 4-h time course after treatment with 10 Gy in this and two other independent experiments.

DNA Damage-induced Transcription of *gadd45*, *p21*, and *gadd153*. Wild-type and scid cells were treated with MMS, UV, or IR, and total RNA was harvested 4 h after treatment (Fig. 4). Northern blot analysis of total RNA was used to monitor the expression of several genes known to be induced by DNA damage: *gadd45*, *p21*, and *gadd153*. The blot was first probed for β-actin to confirm equal loading of mRNA (Fig. 4*D*). The membrane was then stripped and rehybridized to a probe for *gadd45*, which is induced after IR by a p53-dependent pathway and after UV by a p53-independent pathway (4, 40). Wild-type and scid early passage fibroblasts showed the same induction pattern: they induced *gadd45* very strongly with MMS treatment and only weakly with UV (40 J/m^2) or IR treatment (10 Gy; Fig. 4*A*). We observed the same induction for a range of UV doses (10–50 J/m^2) and a slightly increased induction when the IR dose was

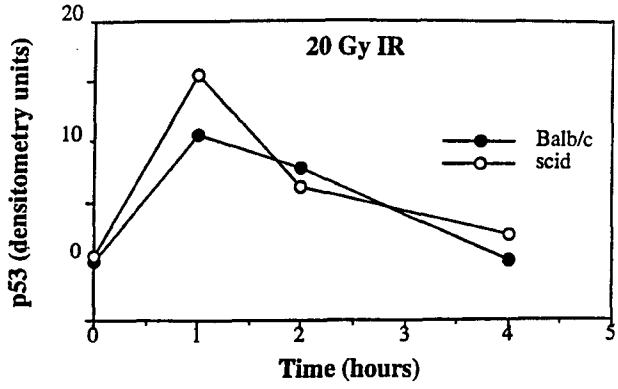

Fig. 3. scid cells accumulate p53 with a normal time course after IR damage. Early passage fibroblasts from wild-type BALB/c and scid mice were treated with 10 Gy IR, and cell lysates were harvested after 0, 1, 2, or 4 h. The accumulation of p53 was detected using Western blot with p53 antibody 240 and quantitated by densitometry.

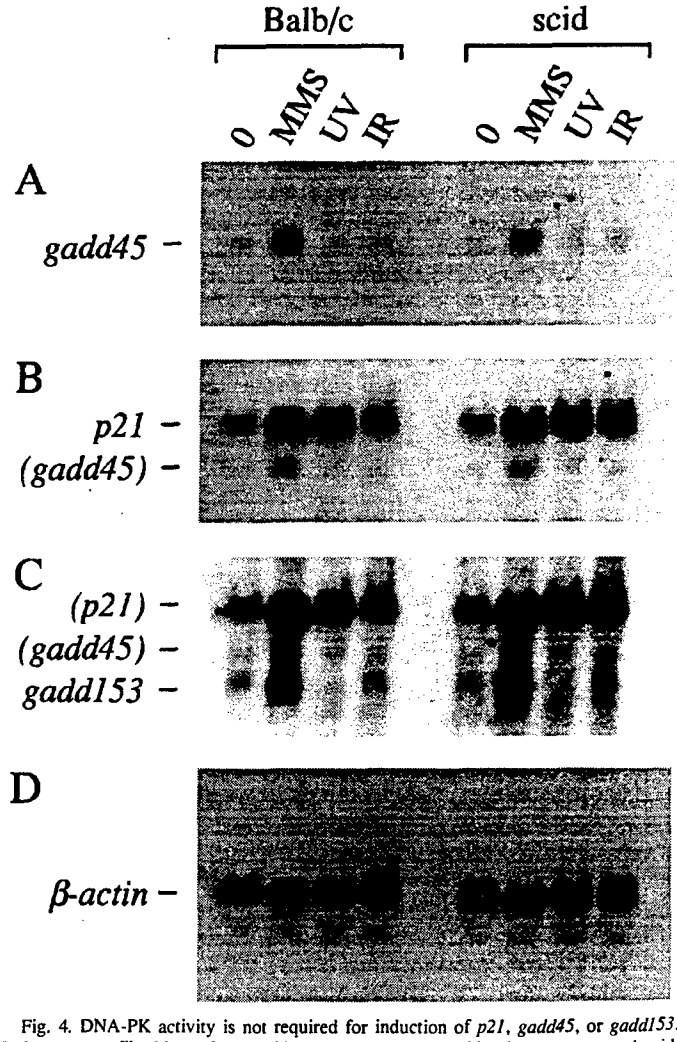

Fig. 4. DNA-PK activity is not required for induction of *p21*, *gadd45*, or *gadd153*. Early passage fibroblasts from wild-type BALB/c and scid mice were treated with DNA-damaging agents, and total RNA was harvested 4 h later. Northern blots were probed for expression of *gadd45* (*A*), *p21* (*B*), *gadd153* (*C*), and β-actin (*D*). Cells were treated as follows: 0, no treatment; MMS, 100 μg/ml; UV, 40 J/m^2 UV radiation; and IR, 10 Gy IR. The blot was first probed with β-actin as a control for equal loading of mRNA, then stripped, and probed seqentially for *gadd45*, *p21*, and *gadd153*. Bands corresponding to each of these genes are indicated on the *left*, and the residual bands from previous probings are indicated in *parentheses*.

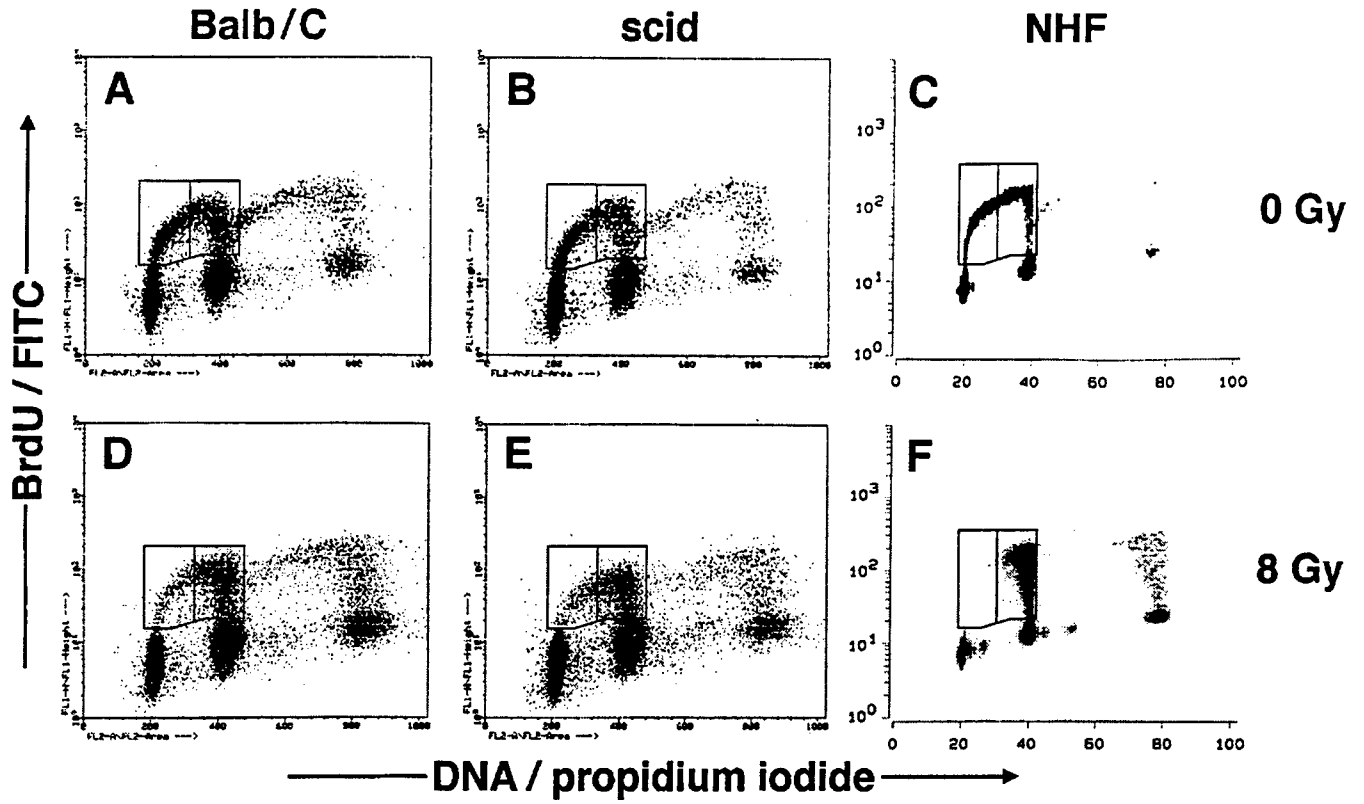

Fig. 5. G_1 checkpoint is intact in scid cells. Asynchronously dividing early passage fibroblasts were mock treated (*A–C*) or exposed to 8 Gy IR (*D–F*). Data are shown for wild-type BALB/c (*A* and *D*); scid (*B* and *E*); and NHFs (*C* and *F*). Cells were analyzed by flow cytometry, which reveals the distribution of nuclei in G_0-G_1, S-phase, and G_2-M for diploid and tetraploid cells. The first and second halves of the S-phase for diploid cells are marked by the *boxes* (first half, *left box*; second half, *right box*). The G_1 checkpoint response was quantitated for Table 1 by measuring selective depletion of the first half of the S-phase compartment (35). Data for NHFs were obtained by staining the cells using the same methods used for the mouse cells, but appear with different axes because a different flow cytometer was used for that particular experiment.

raised to 20 Gy (data not shown). Again, no significant difference was observed in scid and wild-type cells.

The *p21* gene is induced after several forms of DNA damage by a p53-dependent pathway (6, 8, 41). Although the expression of p21 can be regulated in the absence of p53 (43), its induction after exposure to IR appears to be dependent on the presence of wild-type p53 (6, 42). Wild-type and scid cells induced *p21* strongly in response to MMS, UV, and IR (Fig. 4*B*). Dose-response experiments demonstrated increasing *p21* induction with increasing doses of UV (10–50 J/m^2) and the same induction of *p21* for a range of IR doses (10–20 Gy) (data not shown). Again, no significant difference in *p21* induction was observed in scid and wild-type cells.

The *gadd153* gene is induced by UV and MMS but not by IR in Chinese hamster ovary cells (11). Additional studies have linked *gadd153* induction to a p53-independent UV response (44). In our experiments with primary mouse fibroblasts, *gadd153* was strongly induced with MMS, only weakly induced in response to IR, and (in contrast to Chinese hamster ovary cells) weakly suppressed by UV treatment (Fig. 4*C*). Again, no significant difference was observed in scid and wild-type cells.

G_1 Checkpoint Response. Although scid cells had a normal damage response in the specific factors that we examined (p53, p21, gadd45, and gadd153), it remained possible that the physiological cell cycle response might nevertheless be abnormal. Therefore, the G_1 checkpoint response was quantified in early passage cultures of newborn mouse skin fibroblasts using flow cytometry (35). Cells were irradiated with 8 Gy IR to trigger G_1 arrest and then incubated with BrdUrd 6–8 h after irradiation for quantification of the S-phase cells. As demonstrated with the wild-type BALB/c fibroblasts (Fig. 5*A* and *D*), the G_1 checkpoint arrests progression of G_1 cells into the S-phase. Cells that were in the S-phase at the time of irradiation continue progression albeit at a reduced rate. As a result of the inhibition of entry into the S-phase, the S-phase compartment is depleted after irradiation. By 6–8 h after irradiation of the wild-type BALB/c fibroblasts, the fraction of BrdUrd-labeled cells in the first half of the S-phase (2–3N DNA content) was reduced by 67% for BALB/c and by 57% for agouti wild-type cells (Fig. 5 and Table 1). Irradiation of the scid fibroblasts reduced the fraction of diploid cells in the first half of the S-phase by 72% (Fig. 5, *B* and *E*), a value similar to that seen for the wild-type fibroblasts and consistent with an intact G_1 checkpoint.

Notably, NHFs were more sensitive than the three mouse skin fibroblast strains to radiation-induced G_1 arrest. In parallel experiments with human fibroblasts from three different neonates, 8 Gy produced 96% G_1 arrest (Fig. 5, *C* and *F*, and Table 1) and 1.5 Gy produced 75–93% G_1 arrest. With the mouse fibroblasts, G_1 arrest could not be detected after 1.5 Gy, necessitating use of the higher 8-Gy dose.

Table 1 *G_1 checkpoint response after IR in scid and wild-type cells*

The cells were analyzed for G_1 arrest 6–8 h after exposure to 8 Gy IR. The relative numbers of diploid cells in the first (2–3N) and second (3–4 N) halves of the S-phase and depletion of the cells in the first half of the S-phase (G_1 arrest) are shown. The data for 2–3 n and 3–4 n are given as a percentage of mock-treated control cells. Cells analyzed were wild-type BALB/c and agouti mouse cells, scid mouse cells, and NHFs. The numbers of independent experiments are shown in parentheses.

Cells	2–3 N (%)	3–4 N (%)	G_1 arrest (%)
BALB/c (n = 2)	33	112	67
Agouti (n = 2)	43	106	57
Scid (n = 3)	28	103	72
Human (n = 1)	4	152	96

G_2 Checkpoint Response. Mammalian cells also have a G_2 checkpoint that is activated after DNA damage. The G_2 checkpoint was assessed by measuring inhibition of mitosis after IR. Low doses of IR cause G_2 cells to delay entry into mitosis without affecting completion of mitosis (35, 36). Consequently, the fraction of cells in mitosis drops following IR (Fig. 6). After a transient delay, the cells restored progression from G_2 into M and mitosis recovered. The recovery in scid cells was delayed compared to BALB/c cells. This, however, is consistent with the scid repair defect, which leads to persistent DNA double-strand breaks. For both BALB/c and scid cells, inhibition of mitosis was maximal 2 h after treatment with 2 Gy IR. Therefore, the G_2 checkpoint was quantified 2 h after irradiation for additional cell lines: agouti wild-type mouse cells and NHFs as well as BALB/c and scid cells. The scid and wild-type mouse cells displayed the same degree of inhibition of mitosis (Table 2): 78–86% after 2 Gy and 99–100% after 4 Gy. As with the G_1 checkpoint, the G_2 checkpoint response of mouse fibroblasts appeared to be attenuated in comparison to human skin fibroblasts, in which mitosis was inhibited by 99.7% 2 h after treatment with 1.5 Gy IR.

DISCUSSION

DNA-PK has a number of properties that have made it a candidate for transducing DNA damage into a signal for p53 accumulation and cell cycle arrest. Its Ku subunit binds to DNA double-strand breaks, which are induced by IR, and to DNA nicks, which appear as intermediates during the nucleotide excision repair of bulky adducts. It is already known to be involved in the repair of DNA double-strand breaks and therefore has a role in the cellular response DNA damage. The kinase activity is activated by Ku binding to the damaged DNA, and it acts on p53 as well as other proteins involved in regulating the cell cycle. Phosphorylation of p53 induces a conformation change that activates its transcriptional activity (45). The kinase domain of DNA-PK is also homologous to the *ATM* gene, which is known to signal p53-mediated cell cycle arrest (4, 6). Therefore, it was important to test carefully the possible role of DNA-PK in cellular responses to DNA damage.

scid cells were used to test the role of DNA-PK, since these cells express no detectable kinase activity from DNA-PK (30), contain reduced levels of the catalytic subunit $DNA\text{-}PK_{cs}$ protein (31), and are rescued by yeast artificial chromosomes carrying the *DNA-PK*$_{cs}$ gene (30, 31). The available immortalized scid and wild-type mouse cell lines expressed constitutively high levels of p53, suggesting that such cell lines might have abnormal p53 and cell cycle responses to DNA damage. Therefore, our experiments were performed with early passage skin fibroblasts derived from newborn mice.

Accumulation of p53 protein was normal in scid and wild-type cells after treatment with IR or UV. No differences were seen even when p53 was measured at different times after different doses of the DNA-damaging agents. Similarly, the induction of the *p21*, *gadd45*, and *gadd153* genes occurred normally in scid and wild-type cells after treatment with IR, UV, or MMS. Finally, scid and wild-type cells underwent normal G_1 arrest and G_2 delay in response to IR.

These observations demonstrated that the p53-dependent pathway for signaling DNA damage is intact in scid cells and does not require the kinase activity of DNA-PK. First, there was normal accumulation of p53 in scid cells. Second, there was normal induction of two genes, *p21* and *gadd45*, which are transcriptionally activated by p53. It was important to measure *p21* and *gadd45* induction, because transcriptional activation by p53 depends on both the accumulation and posttranslational conformational changes in p53 (46). The possibility that DNA-PK might affect the function of p53 without affecting its accumulation was ruled out by the normal induction of *p21* and *gadd45*.

The observations also demonstrated that several p53-independent damage signaling pathways do not require DNA-PK. The *gadd45* and *gadd153* genes are induced by UV and MMS independently of p53, and the *p21* gene could potentially be induced by p53-independent pathways. Similarly, G_2 delay following DNA damage does not require p53 (36, 42). Again, no abnormalities were seen in scid cells for each of these p53-independent responses.

There are several possible interpretations of our results. The interpretation that we favor is that DNA-PK does not act on p53 *in vivo* and is not involved in activating the G_1 and G_2 checkpoints after DNA damage. A second possible interpretation is that the scid mutation does not lead to complete loss of function of the *DNA-PK*$_{cs}$ gene. There is evidence that the $DNA\text{-}PK_{cs}$ polypeptide is present in scid cells albeit at reduced levels, although the enzymatic activity is undetectable. Thus, the residual levels of DNA-PK might somehow induce the accumulation and activation of p53 despite the absence of kinase activity in the scid cells. Elucidation of the scid mutation will help address this possibility, which we consider unlikely.

A third possible interpretation is that DNA-PK is involved in cell cycle regulation, but the loss of DNA-PK activity is concealed by the existence of overlapping pathways that activate p53 and induce cell cycle arrest independently of DNA-PK. For example, p53 is activated for sequence specific binding by short single strands of DNA by a mechanism that does not require any other proteins (47). Furthermore, p53 will bind directly to insertion-deletion mismatches, suggesting that it may be capable of acting directly as a sensor for DNA damage (48). The p53 response may also be regulated by the gene that is mutated in ataxia telangiectasia (*ATM*). Ataxia telangiectasia cells are abnormally sensitive to IR and show delayed accumulation of p53 and

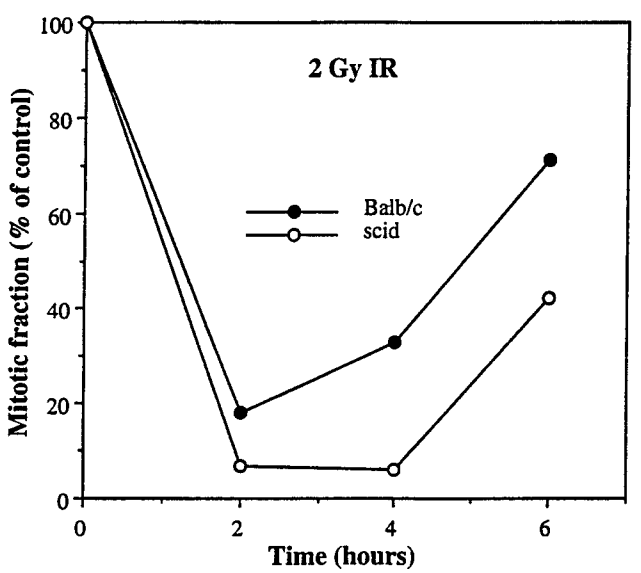

Fig. 6. G_2 checkpoint is intact in scid cells. BALB/c wild-type and scid cells were analyzed for G_2 delay after exposure to 2 Gy IR. The mitotic fraction was assessed at different times following IR. The mitotic fraction observed in the treated cells was then divided by the mitotic fraction observed in the mock-treated control cells and multiplied by 100 to yield a mitotic fraction expressed as a percentage of the control (35).

Table 2 *G_2 checkpoint response after IR in scid and wild-type cells*

The cells were analyzed for G_2 arrest after exposure to 2 and 4 Gy IR. The fraction of cells in mitosis as a percentage of mock-treated control cells 2 h after exposure is shown. The numbers of independent experiments are shown in parentheses.

Cells	2 Gy (%)	4 Gy (%)
BALB/c	22 (n = 2)	0 (n = 1)
Agouti	14 (n = 1)	0 (n = 1)
scid	14 (n = 5)	1 (n = 4)

impaired G_1 arrest (4, 6, 22). Thus, the *ATM* gene acts upstream of p53 in the damage response pathway. The *ATM* gene shares homology with the putative kinase domain in *DNA-PK*$_{cs}$ (21, 49), suggesting that the ATM protein might be functionally similar to DNA-PK in being capable of phosphorylating p53 directly.

Given the possibility that redundant pathways may exist for transducing DNA damage into the p53-dependent response and cell cycle arrest, the data do not completely rule out a role for DNA-PK. However, the experiments do rule out an essential role for DNA-PK in both G_1 and G_2 checkpoint responses and strongly suggest that if there is a role, it must be a minor one. We were unable to find any evidence for an abnormal damage response in scid cells, apart from the known defect in double-strand break repair. The scid cells responded normally after several different forms of DNA damage. Both the magnitude and the time course of the response was indistinguishable from the response in wild-type cells when we measured accumulation of p53, induction of *p21*, *gadd45*, and *gadd153*, and arrest of the cell cycle in G_1. Although G_2 delay was prolonged in scid cells, this result was consistent with the prolonged presence of DNA double-strand breaks in the repair-deficient scid cells and clearly showed that the G_2 checkpoint was intact.

Given the normal response of scid cells when analyzed with respect to so many parameters, we believe that the best interpretation of the data is that DNA-PK is not involved in the p53-dependent response nor in the arrest of the cell cycle after cells are exposed to DNA-damaging agents.

ACKNOWLEDGMENTS

We thank Yan-Ru Chen and Gregory Barsh for guidance in establishing the early passage fibroblast cultures and for the agouti mice; H. Leo Aguila and Irving Weissman for helpful discussions and providing the scid and BALB/c mice; Al Fornace for helpful discussions and for the gadd45 and gadd153 probes; Stephen Elledge for the Cip1 probe; and Lawrence Donehower for the p53−/− cells.

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Reprint from

Current Topics in Microbiology and Immunology, Vol. 217

Molecular Analysis of DNA Rearrangements
in the Immune System

Editors: R. JESSBERGER and M.R. LIEBER

Springer

Role of the Ku Autoantigen in V(D)J Recombination and Double-Strand Break Repair

GILBERT CHU

1 Introduction

All cells have biochemical pathways for repairing DNA double-strand breaks induced by ionizing radiation (X-rays) and oxidative metabolism. Lymphoid cells also have a V(D)J recombination pathway for rearranging B cell immunoglobulin or T cell receptor genes (LEWIS 1994a). V(D)J recombination involves the cleavage of chromosomal DNA and subsequent resolution of the double-strand breaks. In the past few years, it has become clear that the two pathways share a number of common factors.

One of the factors involved in both V(D)J recombination and double-strand break repair (DSBR) was recently identified as the Ku autoantigen (GETTS and

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Stamato 1994; Rathmell and Chu 1994a,b; Smider et al. 1994; Taccioli et al. 1994). Ku was originally identified as the target antigen in a patient with the scleroderma-polymyositis overlap syndrome (Mimori et al. 1981). Because of its association with autoimmune disease, Ku has been extensively characterized over the last 15 years (Reeves 1992).

This paper will examine the biochemical role of the Ku autoantigen in V(D)J recombination and DSBR. To accomplish this, we will review what is known about the genetics of these pathways, the biochemistry of the Ku autoantigen, and the DNA intermediates in V(D)J recombination. We will consider a paradox raised by experiments studying the resolution of DNA hairpins in cells from the severe combined immunodeficient (scid) mouse. To resolve the paradox, we will propose a model for the role of Ku in V(D)J recombination and DSBR.

2 Genetics of V(D)J Recombination and DSBR

2.1 Recombination Signal Sequences and the RAG1 and RAG2 Genes

The germline loci of the immunoglobulin and T cell receptor genes contain multiple V, J, and in some cases D, coding elements. During the development of B and T cells, these elements undergo V(D)J recombination, which involves the rearrangement of V, D, and J elements to form functional genes (Lewis 1994a). The multiplicity of elements generates diversity in the expressed proteins. Rearrangements are targeted to recombination signal sequences (RSS), which consist of conserved heptamer and nonamer sequences separated by a spacer of 12 or 23 base pairs. Coding elements are fused at coding joints to form rearranged genes capable of encoding functional proteins. The coding joints are formed with deletions or insertions that generate additional diversity. Signal elements are fused at signal joints, which are formed precisely with exact conservation of the RSS.

V(D)J recombination is initiated by the combined action of the recombination activating genes, RAG1 and RAG2, which are expressed specifically in lymphoid cells. Cotransfection of RAG1 and RAG2 confers V(D)J recombination activity to nonlymphoid cells (Oettinger et al. 1990; Schatz et al. 1989). Thus, once V(D)J recombination is initiated by RAG1 and RAG2, general factors present in all cells will complete the recombination reaction. These general factors also have a second function in DSBR, as discussed below.

2.2 The Scid Mouse Is Defective in Both V(D)J Recombination and DSBR

The scid mouse lacks mature T and B cells and is highly susceptible to the development of T cell lymphomas (Bosma and Carroll 1991). The failure to develop a competent immune system was explained by the discovery that scid cells cannot form coding joints during V(D)J recombination (Lieber et al. 1988). By contrast, scid cells can form signal joints at a normal rate, although only 50% of the joints are precise.

In addition to a lymphoid-specific defect, scid cells were found to have a general defect in DSBR affecting all tissues and causing hypersensitivity to ionizing radiation due to a deficiency in repairing DNA double-strand breaks (Biedermann et al. 1991; Fulop and Phillips 1990; Hendrickson et al. 1991). This was the first evidence that V(D)J recombination and DSBR utilize common factors present in all tissues.

2.3 Multiple Cell Lines Defective for Both V(D)J Recombination and DSBR

Ionizing radiation produces several different DNA lesions, including base damage, single-strand breaks, and double-strand breaks. To search for the genetic basis of resistance to ionizing radiation, a number of easily cultured hamster cell lines have been developed by mutagenesis and screening for hypersensitivity to ionizing radiation (Jeggo 1990). Cell fusion experiments show that these cell lines fall into at least ten genetic complementation groups. The corresponding genes are designated XRCC1, XRCC2, etc., for X-ray cross-complementing, since early efforts were aimed at cloning the genes by cross-complementation of the X-ray-sensitive hamster cells with human DNA.

The discovery that scid cells are sensitive to X-rays raised the possibility that other X-ray sensitive cells might also be defective in V(D)J recombination. Therefore, cell lines were screened by cotransfection of RAG1, RAG2, and an extrachromosomal V(D)J recombination substrate. Cell lines from each of three complementation groups with defects in DSBR also proved to be defective in V(D)J recombination (Lee et al. 1995; Pergola et al. 1993; Taccioli et al. 1993). Complementation group 7 (which includes scid) was defective for coding joint but not signal joint formation. Complementation groups 4 and 5 were defective for both coding and signal joint formation. The residual recombination events recovered from mutant cells were characterized by abnormally large nucleotide deletions in the coding joints in group 7 or both coding and signal joints in groups 4, and 5. Thus, at least three gene products are involved in a pathway common to both V(D)J recombination and DSBR. The genetics of V(D)J recombination are summarized in Fig. 1.

A possible explanation for the large deletions during the joining reaction was that the cells might be defective in a protein that bound and protected

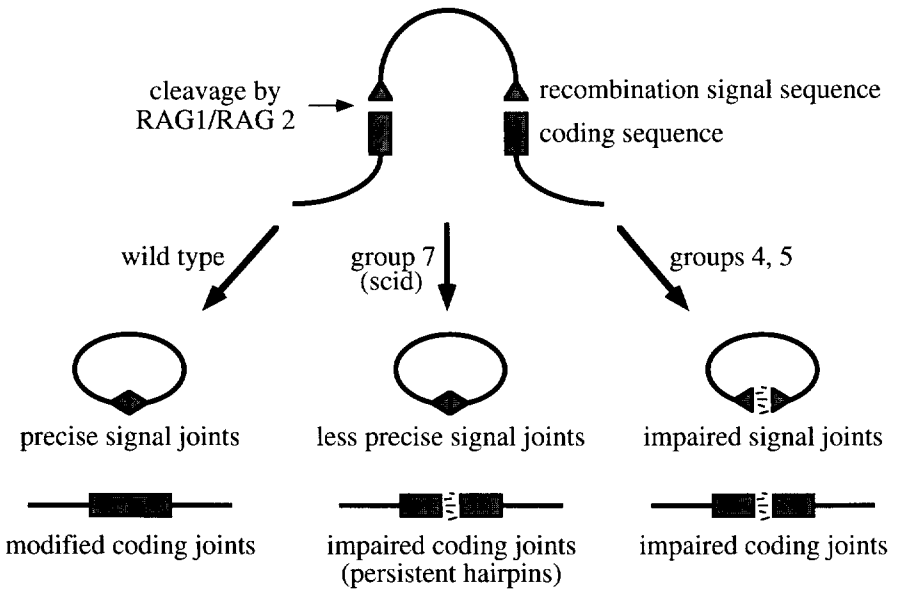

Fig. 1. Genetics of V(D)J recombination. The RAG1 and RAG2 genes induce cleavage adjacent to RSS. In wild-type cells, recombination then produces precise signal joints and modified coding joints. In cells from X-ray sensitivity complementation group 7, which corresponds to the scid defect, coding joints are severely impaired while signal joints are only mildly impaired. The impairment in coding joints is accompanied by the persistence of hairpin ends. In cells from groups 4, and 5, both coding and signal joints are severely impaired

the DNA ends from nuclease degradation. This possibility was confirmed by discovery of the normal role of the Ku autoantigen in cells.

3 The Ku Autoantigen

Ku is the target antigen in patients with several autoimmune diseases, including scleroderma-polymyositis overlap syndrome, sytemic lupus erythematosus, Grave's disease, and Sjögren's syndrome (Reeves 1992). Ku is a highly stable heterodimer of 70 kDa and 86 kDa polypeptides (Ku70 and Ku80) (Mimori et al. 1986). The cDNAs for both subunits have been cloned (Mimori et al. 1990; Reeves and Sthoeger 1989; Yaneva et al. 1989). Ku is localized to the nucleus and moderately abundant, with about 200 000 to 400 000 molecules present in each cell.

Ku has an interesting specificity for its DNA substrates. It does not bind to single-stranded DNA ends, but binds tightly to double-stranded ends, having equal affinity for 5' overhanging, 3' overhanging, and blunt ends (Mimori and Hardin 1986). Ku also binds to DNA nicks (Blier et al. 1992) and to DNA ending in stem loop structures (Falzon et al. 1993). This spectrum of DNA binding

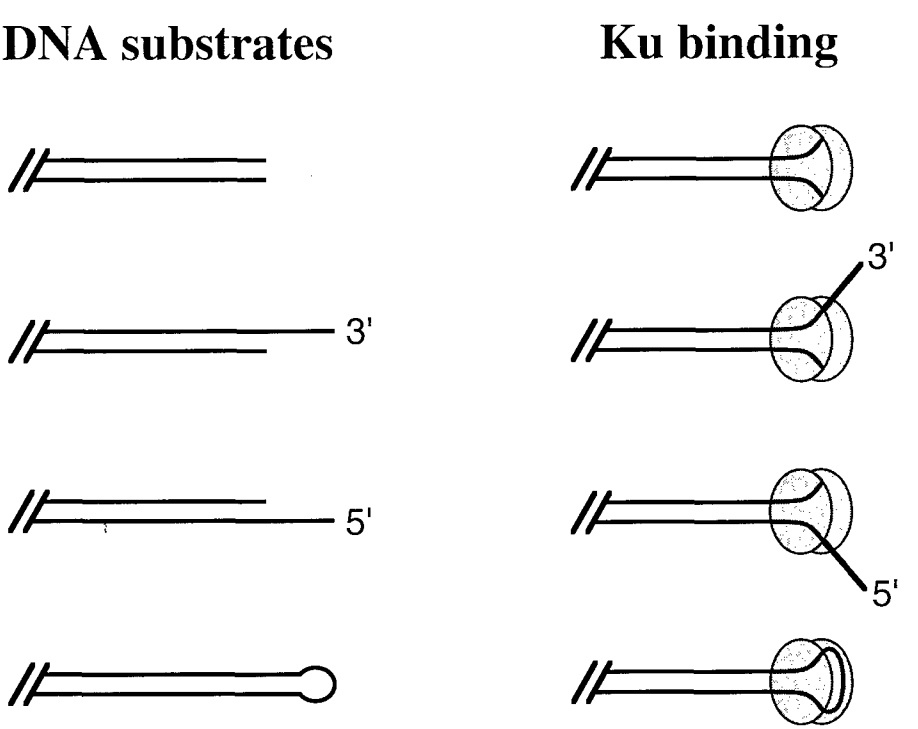

Fig. 2. Model for Ku DEB activity. Ku binds to a number of DNA substrates, including DNA with blunt ends, 5' or 3' overhanging ends, or stem loop structures. Ku may recognize all of these structures by binding to the forked DNA structure formed at the transition of double-stranded DNA to two single strands

activity can be explained by a model in which Ku recognizes transitions between double-stranded DNA and two single strands, as depicted in Fig. 2. Once Ku binds to DNA ends, it is capable of translocating along the DNA, so that three or more molecules of Ku can become bound to a single DNA fragment (PAILLARD and STRAUSS 1991).

Ku is conserved both functionally and structurally across a broad range of eukaryotes. The fruit fly *Drosophila melanogaster* (BEALL et al. 1994; JACOBY and WENSINK 1994) and yeast *Saccharomyces cerevisiae* (FELDMANN and WINNACKER 1993) contain DNA end-binding (DEB) activities arising from heterodimeric proteins of similar molecular weights as mammalian Ku. In both cases, the gene for the smaller (70 kDa) subunit was cloned and found to be homologous to mammalian Ku70. The genome project for the nematode *Caenorhabditis elegans* has revealed an open reading frame encoding a polypeptide homologous to mammalian Ku80.

Ku is the regulatory subunit of an unusual enzyme, DNA-dependent protein kinase (DNA-PK), which also includes an enormous 460 kDa catalytic subunit (DNA-PK_{cs}). DNA-PK is normally inactive in free solution, but when it assembles

on a DNA substrate recognized by Ku, DNA-PK is activated for its kinase activity (Gottlieb and Jackson 1993). Although the in vivo substrates of DNA-PK remain to be defined, potential substrates have been identified in vitro, including Ku70 and Ku80, tumor suppressor protein p53, the C-terminal domain of RNA polymerase II, replication protein A (RPA), topoisomerases I and II, serum response factor (SRF), SV40 T antigen, *Xenopus* histone protein 2A.X, and the transcription factors c-*myc*, c-*fos*, c-*jun*, Sp1, TFIID and Oct-1 (Anderson 1993). DNA-PK will phosphorylate Sp1 when it is bound to the same DNA molecule, even when separated by several kilobases, but will not act on Sp1 bound to a different DNA molecule (Gottlieb and Jackson 1993).

Ku undergoes post-translational modification. When Ku is autophosphorylated by DNA-PK, it acquires an ATPase activity (Cao et al. 1994), making Ku an ATP dependent helicase (Tuteja et al. 1994). When Ku binds to one of its substrates, it recruits DNA-PK_{cs} and then is autophosphorylated to become an active helicase. Ku may also be processed by proteolytic cleavage of the Ku80 subunit (Paillard and Strauss 1993).

4 Identification of Genes Involved in both V(D)J Recombination and DSBR

4.1 DEB Activity in Mutant and Wild-Type Cells

DEB activity can be detected in cells by an electrophoretic mobility shift assay (EMSA) (Rathmell and Chu 1994a). Extracts of the cells are incubated with a labeled linear fragment of DNA in the presence of unlabeled circular plasmid DNA to mask the effect of nonspecific DNA binding proteins. The reaction mixture is resolved by nondenaturing polyacrylamide gel electrophoresis, and DEB activity is detected as an upward shift in electrophoretic mobility of the DNA probe.

DEB activity was found to be present in cells from yeast and from a number of tissues from humans and rodents (Table 1) (Rathmell and Chu 1994a). It was expressed normally in cell lines from groups 1, 4, and 7 (including scid cells), in an ataxia telangiectasia cell line, two bleomycin-sensitive cell lines, and a mutant X-ray sensitive lymphoblast cell line. DEB activity was notably absent in three cell lines from X-ray complementation group 5, XR-V15B, XR-V9B, and xrs5. Azacytidine will induce xrs5 cells to revert to X-ray resistance at a frequency of about 1%. Significantly, 23 independent clones selected for X-ray resistance all showed restored DEB activity, further supporting a role for DEB activity in X-ray resistance.

Table 1. DEB activity in wild-type and mutant cell lines

Cell type	Cell lines	Resistance to IR	Complement group	DEB activity
Yeast				
S. cerevisiae	hdf1/pHDF1	+		+
S. cerevisiae	hdf1	+		–
Human				
Fibroblast (wild-type)	IMR-90	+		+
Ataxia telangiectasia fibroblast	AT5BI	–		+
Mouse				
Skin fibroblast (wild-type)	C.B-17	+		+
Skin fibroblast	scid	–	7	+
Chinese hamster				
Lung fibroblast (wild-type)	V79, V79B	+		+
Lung fibroblast	XR-V15B, XR-V9B	–	5	–
Ovary (wild-type)	AA8	+		+
Ovary	EM9	–	1	+
Ovary	XR-1	–	4	+
Ovary	V3	–	7	+
Ovary	xrs5, xrs6, sxi2, sxi3	–	5	–
Ovary	xrs5(rev)[a]	+		+

DEB activity was measured in yeast, human, and rodent cells by the gel mobility shift assay. DEB activity is specifically absent in complementation group 5. DEB activity is present in several other sensitive cell lines, including ataxia telangiectasia cells and cells from complementation groups 1, 4, and 7 (including scid). The table compiles data from (Boubnov et al. 1995; Lee et al. 1995) for sxi cells, from (Getts and Stamato 1994) for xrs6 cells, and from (Rathmell and Chu 1994a) for the remaining cell lines. The HDF1 gene encodes a 70 kDa subunit of a DEB activity in yeast that is homologous to mammalian Ku70 (Feldmann and Winnacker 1993). The deletion mutant hdf1 is resistant to ionizing radiation (S. Cheng and G. Chu, unpublished results), suggesting that the dominant mechanisms for X-ray resistance differ in yeast and mammals.

[a] xrs5(rev) represents pooled xrs5 cells selected for X-ray resistance after azacytidine treatment.

4.2 DEB Activity Is Due to Ku Autoantigen

DEB activity and Ku share many similarities, including nuclear localization, abundance in the cell, and magnesium-dependent DNA binding (Rathmell and Chu 1994b). Most striking is the absolute concordance of DNA substrates. DEB activity and Ku bind to M13 circular virion DNA (presumably via binding to hairpin loops in M13), double-stranded DNA ends with 5', 3', or blunt ends, but not to the single-stranded DNA ends in poly(dA) and poly(dT). Three or more Ku molecules can load and bind to a single DNA fragment, producing a ladder of mobility shifts similar to that seen with DEB activity.

DEB activity and Ku are antigenically similar (Getts and Stamato 1994; Rathmell and Chu 1994b). When added to binding reactions with hamster and human extracts, Ku antisera from two different autoimmune patients produce a supershift in mobility of the DEB protein–DNA complex. When used to

probe immunoblots, these antisera detect a 70 kDa polypeptide in hamster extracts that cofractionates with DEB activity on heparin agarose. The 70 kDa polypeptide was absent or only barely detectable in three hamster cell lines from group 5, but present in group 4, group 7, and wild-type cell lines (Rathmell and Chu 1994b). Thus Ku70 polypeptide is deficient in group 5 cells.

Despite this observation, Ku70 does not define the genetic defect in group 5. Chromosome mapping studies assigned the Ku70 gene to human chromosome 22q13 and the Ku80 gene to chromosome 2q33-35 (Cai et al. 1994; Boubnov et al. 1995). X-ray resistance in group 5 cells was partially restored by human chromosome region 2q35 (Chen et al. 1994; Hafezparast et al. 1993). This raised the possibility that the primary defect in group 5 resides in the gene for Ku80.

Direct evidence that the XRCC5 gene was identical to the Ku80 gene was provided by DNA transfection experiments. Thus, transfection of group 5 cells with human Ku80 but not Ku70 led to partial restoration of DEB activity, X-ray resistance, and V(D)J recombination (Smider et al. 1994; Taccioli et al. 1994). Full restoration was obtained by the isolation and transfection of the hamster Ku80 cDNA. In addition, Ku70 protein levels were restored, suggesting that the Ku70 polypeptide is stabilized by the presence of normal Ku80 polypeptide (Errami et al. 1995). Finally, Ku80 cDNA from XR-V15B and XR-V9B cells contained in-frame deletions of 46 and 84 amino acids, proving that mutations in the Ku80 gene are responsible for the phenotype of group 5 cells (Errami et al. 1995).

The finding that the Ku80 gene is identical to the XRCC5 gene immediately raised the possibility that DNA-PK_{cs} might be encoded by another XRCC gene involved in DSBR. Yeast artificial chromosomes carrying the DNA-PK_{cs} gene will rescue group 7 cells, making DNA-PK_{cs} a candidate for XRCC7 (Blunt et al. 1995; Kirschgessner et al. 1995). Recently, a novel cDNA has been isolated as a candidate for XRCC4 by complementation of XR-1 cells (see Li et al. 1995; Z. Li and F. Alt, this volume). No Ku70 mutant has yet been confirmed.

These experiments demonstrate that V(D)J recombination and DSBR include an overlapping pathway. Furthermore, at least four different genes act in the overlapping pathway (Table 2). Together with recent progress in identifying the enzymatic activities of RAG1 and RAG2, it is now possible to construct a model for the biochemistry of V(D)J recombination. Before introducing the model, it will be necessary to examine the DNA intermediates in V(D)J recombination.

Table 2. Genes involved in V(D)J recombination

Gene	Mutant cell lines	IR	V(D)J activity	
			Coding joints	Signal joints
Specific for V(D)J				
RAG1	KO mouse	R	No cleavage	No cleavage
RAG2	KO mouse	R	No cleavage	No cleavage
TdT	KO mouse	R	No N-addition	Normal
General for DSBR				
XR-1 (XRCC4)	XR-1	S	Deficient	Deficient
Ku80 (XRCC5)	xrs5, xrs6, sxi2, sxi3, XR-V15B, XR-V9B	S	Deficient	Deficient
Ku70 (XRCC6)	sxi1 ?	S ?	Deficient ?	Deficient ?
DNA-PK_{cs} (XRCC7)	scid mouse, V-3	S	Deficient	Normal rate, partial fidelity

The genes involved in V(D)J recombination fall into two classes: those specific for V(D)J recombination and those general for DSBR. Some of the mutant cell lines were generated from mice with the scid mutation or with targeted knockout (KO) of the RAG1, RAG2, and TdT genes. The remaining cell lines were generated from mutagenesis of Chinese hamster cells. Cell lines mutant for genes specific for V(D)J recombination are resistant (R) to ionizing radiation (IR), whereas the cell lines mutant for genes general for DSBR are sensitive to ionizing radiation and fall into X-ray complementation groups 4, 5, or 7. Group 6 has been reserved for Ku70 (XRCC6), but no cell line has been found yet for this group.

5 DNA Intermediates in V(D)J Recombination

5.1 Formation of Broken DNA Ends

The association between DSBR and V(D)J recombination suggests that V(D)J joining involves DNA intermediates in which both strands have been broken. Such broken molecules have been observed directly. Thymocytes from newborn mice actively rearrange the T cell receptor locus and contain broken DNA with blunt signal ends (Roth et al. 1992). On the other hand, coding ends are not detectable, even though a primary double-strand break should liberate one coding end for each signal end.

In fact, coding and signal joints are formed differently. Signal joints are formed without the addition or loss of nucleotides. Coding joints often contain either deletions or short insertions of extra nucleotides not present in the germline DNA. In some cases, the insertions have short palindromic sequences derived from one of the coding ends (Lafaille et al. 1989; McCormack et al. 1989). These P (palindromic) insertions are potentially explained by a model in which coding ends are created by the formation of a hairpin intermediate (Lieber 1991). If the hairpin is nicked at a position away from the tip, a palindromic sequence appears in the completed coding joint. The first direct evidence for hairpin ends was found in scid thymocytes (Roth et al. 1992). Hairpin

ends were not found in wild-type thymocytes, suggesting that coding joints are formed much more rapidly than signal joints. Thus, the scid defect appears to disrupt hairpin processing, allowing hairpin ends to accumulate to detectable levels in scid but not wild-type thymocytes.

Hairpin ends are created as part of the V(D)J cleavage reaction (see Fig. 3). In an experimental tour de force, purified recombinant RAG1 and RAG2 proteins were recently shown to directly catalyze a double-strand break in DNA molecules containing a recombination signal sequence (VAN GENT et al. 1995). The reaction is absolutely dependent on the RSS and both RAG1 and RAG2. Cleavage occurs in a two-step reaction in which RAG1 and RAG2 first nick the DNA 5′ to the RSS; after a time delay, RAG1 and RAG2 catalyze a nucleophilic attack by the 3′ OH of the opposite strand (MCBLANE et al. 1995). This second step generates a blunt signal end and a hairpin coding end.

The coding ends are subjected to further modification by nucleases (N deletion) or by the nontemplated addition of nucleotides (N insertion). Most N addition occurs by 3′ addition catalyzed by terminal deoxynucleotidyl transferase (TdT), which is expressed only in lymphoid cells. A low rate of N insertion occurs in all cells, perhaps by the capture of free nucleotides or oligonucleotides (ROTH et al. 1989).

5.2 Joining Reactions

The rejoining reaction in V(D)J recombination does not require extensive homology in the recombining DNA. V(D)J recombination will still occur in extrachromosomal substrates with homopolymeric coding sequences that do not permit homologous pairing (BOUBNOV et al. 1993).

On the other hand, V(D)J recombination preferentially utilizes short stretches of homology if they are present. When nonlymphoid cells are cotransfected with RAG1, RAG2, and an extrachromosomal substrate, the majority of coding joints are formed at positions containing short homologies of 1–5 bp. In extrachromosomal substrates constructed with 4 bp of homology at the two coding ends, there was a strong bias towards the coding joint formed by homology alignment of the 4 bp repeats (GERSTEIN and LIEBER 1993).

In lymphoid cells, homology pairing may be obscured by TdT activity, since N insertion can include homologous nucleotides that would disappear once the joint is made. In lymphoid cells from TdT knockout mice, N insertion is virtually eliminated (GILFILLAN et al. 1993; KOMORI et al. 1993). In the absence of TdT, homology alignment occurs in 75% of the coding joints. Thus, the use of homology is preferred, but not essential for the formation of coding joints. Homology affects the distribution of coding joints but not the overall efficiency of the reaction.

General DSBR has been studied by transfecting linearized SV40 DNA into nonlymphoid cells (ROTH et al. 1985; ROTH and WILSON 1986). These plasmids did not contain RSS and were linearized by cutting the SV40 genome in a

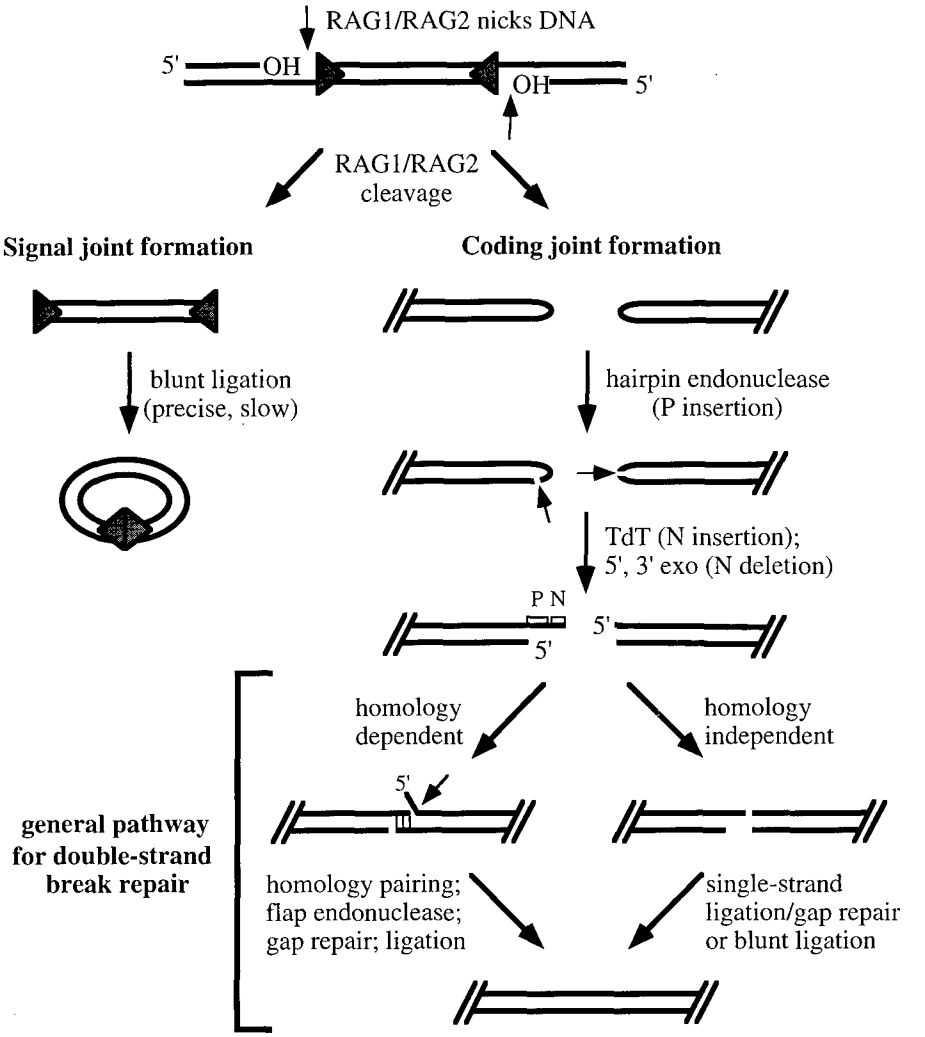

Fig. 3. DNA intermediates during V(D)J recombination. RAG1 and RAG2 recognize RSS (*shaded triangles*) and nick the adjacent DNA, leaving a 3' hydroxyl group. RAG1 and RAG2 then catalyze a nucleophilic attack by the 3' OH of the opposite strand to complete the cleavage reaction, leaving a blunt signal end and a hairpin coding end. A hairpin endonuclease then opens the hairpin symmetrically to leave a blunt end, or asymmetrically to leave an overhanging end that potentially leads to a palindromic sequence at the coding joint (P insertion). Nucleotides may be added (N insertion) by terminal deoxynucleotidyl transferase (TdT) or deleted (N deletion) by the activity of exonucleases. The ends are joined by homology-dependent or homology-independent mechanisms. Homology-dependent joining may involve pairing in regions of microhomology, removal of unpaired end fragments by a flap endonuclease, repair of gaps by DNA polymerase, and ligation of the nicks. Homology-independent joining involves either single-strand ligation and gap repair or blunt ligation

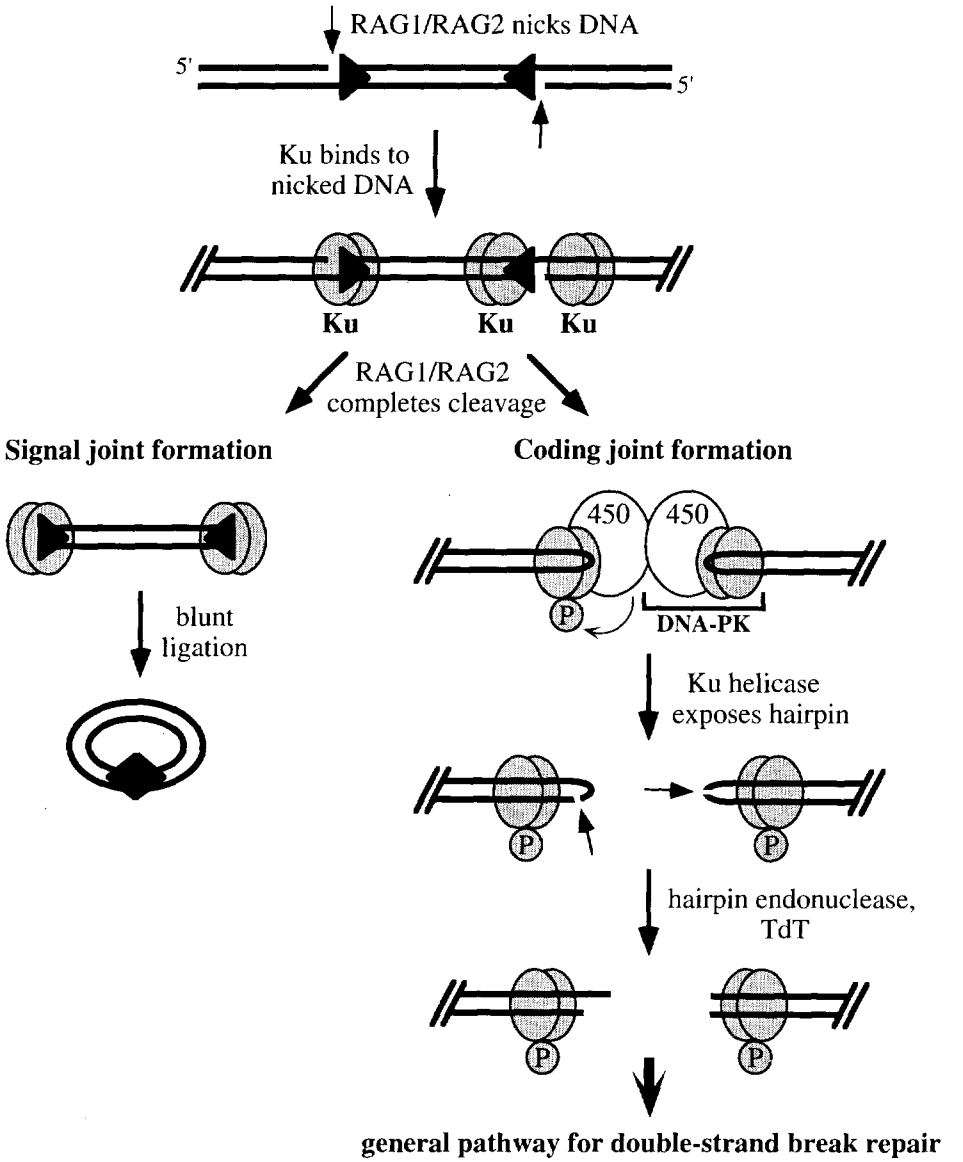

Fig. 4. Model for Ku in V(D)J recombination. After RAG1 and RAG2 nick the DNA at the RSS (*shaded triangles*), Ku (*shaded dimer*) binds to the nicked DNA. Therefore, when cleavage is completed, Ku is immediately loaded onto the newly created signal and coding ends. The signal joint is dependent on Ku but not DNA-PK_{cs}, the 460 kDa catalytic subunit of DNA-PK. The coding joint is formed by processing of the hairpin ends. DNA-PK is assembled by recruitment of DNA-PK_{cs} to the hairpin end. DNA-PK_{cs} has an alignment function to bring the DNA ends in proximity to each other. DNA-PK autophosphorylates Ku (*encircled Ps*), conferring ATP dependent helicase activity to Ku. The helicase activity unwinds the hairpin, allowing hairpin endonuclease to nick the hairpin. Nucleotides may be added by TdT or deleted by exonucleases. The free ends are then joined by the general pathway for DSBR

nonessential intron, leaving mismatched ends. As for the case of V(D)J recombination, joining occurred by both homology-dependent and homology-independent mechanisms. Homology-independent joining appears to utilize single-strand ligation preferentially over blunt ligation (ROTH and WILSON 1986). This general pathway for DSBR is shown at the bottom of Fig. 3.

In summary, the joining reactions in V(D)J recombination and general DSBR share a common pathway. Both reactions are mediated by the same proteins, namely, Ku, DNA-PK_{cs}, and the XR-1 gene product. Both reactions involve similar DNA intermediates with joining reactions both dependent and independent of microhomology. Pairing by microhomology suggests a mechanism for unwinding the DNA ends, perhaps catalyzed by Ku helicase activity.

6 A Paradox of Hairpin Processing in Scid Cells

The persistence of hairpin ends in scid thymocytes suggests that the scid mutation leads to defective processing of hairpin intermediates during V(D)J recombination. However, when cells were transfected with linearized plasmid DNA carrying hairpin ends, the rejoining reaction occurred with the same efficiency in scid and wild-type pre-B cells (LEWIS 1994b). The joint sequences showed patterns of deletion, P insertion, N insertion, and microhomology usage that were similar in the scid and wild-type cells and similar to the coding joints generated by bona fide V(D)J recombination. Thus we are faced with an apparent experimental paradox: scid cells cannot join hairpin ends created during V(D)J recombination, but can process and join hairpins introduced into the cells by transfection.

7 Solution to the Scid Paradox: A Model for the Role of Ku

7.1 Model for Ku in V(D)J Recombination and DSBR

To explain the DNA intermediates in terms of the biochemical properties of the Ku protein, we propose a model for the role of Ku in V(D)J recombination (see Fig. 4). In this model, the reaction proceeds in the following steps.

1. Concerted action of RAG1 and RAG2 introduces a nick 5' to the signal sequence, allowing Ku to bind to the nicked DNA intermediate. RAG1 and RAG2 then complete the cleavage reaction to form a hairpin coding end and a blunt signal end.

2. Upon creation of the hairpin end, Ku binds immediately because it has been preloaded onto the nicked DNA intermediate.
3. Activated DNA-PK is formed by the recruitment of DNA-PK_{cs} to form a complex with Ku bound to the DNA. DNA-PK_{cs} may serve an alignment function, so that two molecules of DNA-PK_{cs} interact with each other to bring the appropriate coding ends in proximity to each other.
4. DNA-PK autophosphorylates its Ku subunit, conferring to Ku an ATP-dependent helicase activity. (Steps 3 and 4 may precede step 2, since they can occur once Ku is loaded onto the nicked DNA intermediate.)
5. Ku then unwinds the hairpin while moving inward from the end, exposing the hairpin and allowing the hairpin endonuclease to nick and open the hairpin.
6. Ku helicase unwinds the free DNA ends to allow the denatured single strands to align and pair in regions of microhomology.
7. In cases where pairing involves internal sequences, a DNA flap would be created from DNA distal to the region of homology. In fact, a flap endonuclease (FEN-1) has been characterized and purified (HARRINGTON and LIEBER 1994, 1995). FEN-1 specifically recognizes 5' flap structures and cuts precisely at the base of the flap to leave a ligatable nick.
8. Alternatively, the DNA ends are aligned end to end and joined by either single-strand ligation followed by gap filling or blunt ligation. Thus, the joints can be made with or without microhomology. (In steps 6, 7, and 8, the opened hairpin is rejoined in a reaction identical to that used for general DSBR, as shown in Fig. 5.)
9. The two signal ends are brought together to form a signal joint in a reaction that requires Ku and the XR-1 gene product but not DNA-PK_{cs}. The rate of signal joint formation is likely to be much slower than for coding joints, since signal ends but not coding ends are readily detectable in wild-type thymocytes. Delayed formation of signal joints may be a mechanism for suppressing the unproductive fusion of a coding end to a signal end. Such hybrid joints are less likely if coding joints are already formed before signal joining begins.

7.2 Resolving the Scid Paradox

The scid paradox is resolved by the proposed model for Ku. In the model, the hairpin endonuclease gains access to transfected hairpins but not to V(D)J hairpins. In the case of transfected DNA, the hairpin endonuclease would nick the hairpin before Ku has a chance to bind, explaining the proper processing of transfected hairpins in scid cells. In the case of V(D)J recombination, the two step cleavage reaction by RAG1 and RAG2 first creates a nick adjacent to the signal sequence, allowing Ku to be loaded onto the DNA. When the hairpin is created, Ku is already present, thus denying access to the hairpin endonuclease. In scid cells deficient for DNA-PK_{cs}, Ku would fail to acquire

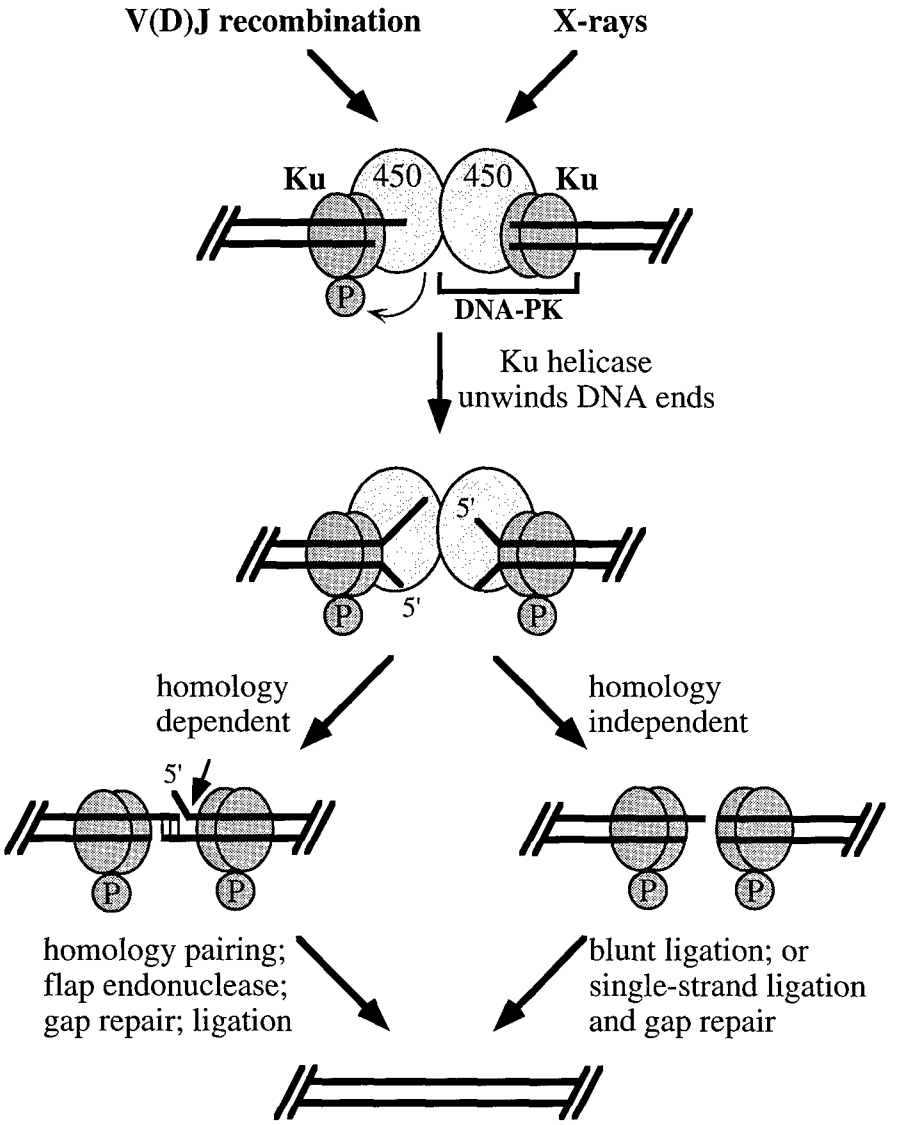

Fig. 5. Model for Ku in DSBR. Free DNA ends are created by X-rays or as intermediates in V(D)J recombination. DNA-PK_{cs} has an alignment function and autophosphorylates Ku to give Ku helicase activity. Ku helicase unwinds the DNA ends, which can then be joined by homology dependent or homology independent mechanisms (see Fig. 3). The overall joining reaction is non-conservative and may involve the loss of nucleotides

helicase activity and continue to protect the hairpin end, explaining the persistence of hairpin ends in scid thymocytes. It is noteworthy that the rare coding ends recovered from scid cells show abnormally large P insertions, suggesting that the hairpin endonuclease was denied access to the tip of the hairpin but could gain access to DNA beyond the site of Ku binding.

Although transfected hairpin DNA is resolved with normal efficiency in scid cells, our model for Ku predicts that fine structure in the processing of the hairpins must be different in scid cells. Once the hairpin is opened into a free DNA end by the hairpin endonuclease, Ku would bind to the DNA end. However, since DNA-PK_{cs} is defective in scid cells, Ku would not become phosphorylated and would not acquire helicase activity. As a result, alignment by microhomology should be reduced compared to wild-type cells. In fact, data from transfection of hairpin molecules into pre-B cells (LEWIS 1994b) suggest that this might be true. Ku helicase activity should affect only joints in which nucleotides are lost. When the data are re-examined for joints in which three or more nucleotides are lost from one or both hairpin ends, there appears to be a difference between scid and wild-type cells. In scid cells, only one of nine joints showed significant loss of nucleotides and concomitant evidence for alignment by microhomology, which consisted of only 2 bp and therefore could have been due to fortuitous exonuclease degradation of a DNA end rather than helicase mediated unwinding. By contrast, in wild-type cells, six of nine joints showed significant loss of nucleotides and evidence for alignment by microhomology, utilizing homologies of 1, 2, 2, 3, 4, and 4 bp. Of course, the number of joints analyzed in this experiment was small and conclusive evidence must await analysis of a larger set of joints. Nevertheless, the joints formed in scid cells appeared biased against nucleotide loss and microhomology utilization, consistent with an inactive helicase unable to unwind the free coding ends.

8 Unresolved Questions for the Role of Ku in V(D)J Recombination

Although the proposed model explains the scid paradox, a number of features must be tested. The DNA-PK_{cs} polypeptide is required for V(D)J recombination and DNA-PK autophosphorylates Ku in vitro, but it is not yet known whether: (1) the kinase activity of DNA-PK affects V(D)J recombination; (2) autophosphorylation of Ku occurs in the intact cell; or (3) Ku helicase activity is required for hairpin processing and subsequent coding joint formation.

If Ku helicase activity is required for V(D)J recombination, such unwinding activity might need to be suppressed for signal joint formation, which must occur precisely without loss or addition of nucleotides. Several possible mechanisms can be envisioned: (1) Ku could bind more tightly to a DNA end adjacent

to a RSS; (2) Ku bound to the signal end might not be accessible to phosphorylation because of binding by the RAG1-RAG2 complex to the RSS at the signal end; (3) Ku helicase activity is rather weak in a standard helicase assay (TUTEJA et al. 1994), and may not be robust enough to unwind a blunt signal end.

There seems to be an extravagant excess of Ku, with about 400 000 molecules per cell. In lymphoid cells actively undergoing V(D)J recombination, only a few double-strand breaks are created. Because multiple molecules of Ku can load onto either nicked or broken DNA (PAILLARD and STRAUSS 1991), this raises the question of whether a large number of Ku molecules participate in each recombination reaction. One possible model is that Ku cooperates with DNA-PK_{CS} in alignment of free DNA ends. For example, Ku molecules could coat the DNA between two recombination sites. Two DNA-PK_{CS} molecules could be paired with each other while being passed from one Ku molecule to the next until they form a more stable complex with Ku bound to the DNA ends, thus bringing the ends into alignment. This idea is consistent with the finding that DNA-PK_{CS} is much less abundant than Ku in the cell.

DNA-PK_{CS} could perform other functions not yet identified with specific proteins. These functions include those for a hairpin endonuclease or for an alignment protein to facilitate microhomology pairing. Microhomology pairing involves only 1–5 bp and might require an alignment protein for stable duplex formation between the two ends. Either of these functions would make DNA-PK_{CS} important for coding but not signal joints.

The possible role of the XR-1 gene product remains undefined. Since XR-1 is required for both coding and signal joints, it may have an alignment function either independently or in cooperation with DNA-PK_{CS}. Alternatively, it may encode a single-strand ligase, which potentially acts at both coding and signal joints.

9 Conclusions

We have described the experimental evidence that Ku is involved in DSBR and V(D)J recombination. This discovery has stimulated interest in how Ku might be acting in these recombination and repair pathways. We have therefore reviewed the biochemical properties of Ku and the molecular properties of the DNA intermediates in V(D)J recombination. Paradoxically, scid cells process hairpin ends introduced by DNA transfection but fail to process hairpins created during V(D)J recombination. To resolve the scid paradox, we have proposed a model for the role of Ku in V(D)J recombination and DSBR. The hypothesis can be tested experimentally, and the results of such experiments promise to improve our understanding of these critical pathways.

Acknowledgments. The author thanks Rachel Gerstein, David Schatz, Kim Rathmell, and Vaughn Smider for helpful discussions. Supported in part by a grant from the Department of the Army, USA (DAMD17-94-J-4350).

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Rec'd 1/11/2000

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 4 Jan 00

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCA, 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for the attached Grants. Request the limited distribution statements for Accession Document Numbers listed be changed to "Approved for public release; distribution unlimited." This report should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by email at Judy.Pawlus@amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

94-J-4350 AD-B225248

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